

Preparing and Cloning  
A Natural Killer Cell  
Hybridoma

by

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in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2000

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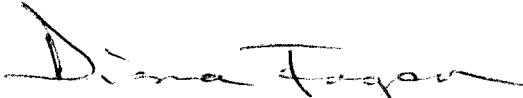
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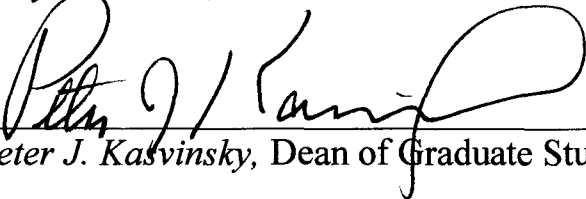
  
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## ABSTRACT

NK cells, a component of our innate immune system, are responsible for the elimination of virus-infected and tumor cells which bypass detection by T cells. In these studies, we attempted to create a NK cell hybridoma clone, which would provide a testable pool of NK cells. Mouse splenocytes were fused with P3X mouse non-secreting myeloma cells in the presence of polyethylene glycol (PEG). Following HAT selection, the cells were tested for the presence of the mouse NK cell surface antigen DX5. After labeling with biotinylated anti-mouse DX5 and Streptavidin-FITC in the presence of rat anti-mouse CD16/CD32 (Fc Block), the mixed lymphocyte cell pool which yielded the highest amount of mean fluorescence ( 257.17 ), was cell pool C6. C6 was then subcloned, and the clones were retested for the presence of DX5 and absence of CD3. The data analysis, however, was hindered by the presence of autofluorescence in the hybridoma cell line. In an attempt to correct the autofluorescence of the hybridomas, two permeabilizing detergents, n-Octyl-B-D-Glucopyranoside (OG), and Cytoperm/Ctyofix™ were used with various concentrations (.01 µg/ml to 10 µg/ml) of the quenching dye trypan blue. Because of the low binding affinity of the anti-DX5 antibody and the inability to compensate for cellular autofluorescence a pure NK cell hybridoma clone could not be identified.

## ACKNOWLEDGEMENTS

I would like to thank my parents, Nicholas and Barbara Damico, and my brother, Jim, for being a constant source of support throughout these past few years. I would not be the person that I am today without the love, guidance and support that my family has given me over the years.

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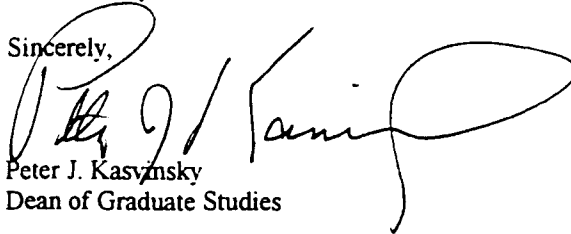
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Department of Biological Sciences  
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Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the Protocol Update you submitted for Approved Protocol #03-97 "Natural Killer Cell Hybridoma Preparation," and has issued ongoing approval for the period of December 10, 1999 through December 10, 2000.

You must adhere to procedures described in your approved request; any modification must first be authorized by the Animal Care and Use Committee.

Sincerely,

  
Peter J. Kasvinsky  
Dean of Graduate Studies

PJK:cc

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## LIST OF ABBREVIATIONS

ADCC	Antibody dependant cell cytotoxicity
APC	Antigen presenting cell
CD	Cluster of differentiation
CTL	Cytolytic T cell
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
HAT	Hypoxanthine aminopterin thymidine
HCL	Hydrochloric acid
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
Ig	Immunoglobulin
MHC	Major histocompatibility complex
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
NK	Natural killer
PBS	Phosphate buffered saline
PE	Phycoerythrin
PEG	Polyethylene glycol
SSC	Side scatter
TCR	T cell receptor
TH	T helper cell
TNF	Tumor necrosis factor

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## I. INTRODUCTION

There are two basic ways in which our body fights off infection. Foreign particles, or antigens, are combated via innate or specific immune responses. The innate immune system provides an early defense against invasive attacks. Innate immunity acts as the body's first line of defense against antigens. There are four main features of the innate immune system (Table 1): 1. natural physical and chemical barriers which ward off infection, 2. blood proteins that serve as barriers to antigenic infection, 3. monocytes and neutrophils eliminate foreign particles via phagocytosis, and 4. non-specific cytotoxic cells called Natural Killer (NK) cells. The innate defenses are non-specific and can protect against a variety of antigens with equal efficiency. However, the innate immune response is not able to inhibit the growth of all pathogens.

The specific immune response has the flexibility to allow protection against a wide variety of pathogens. The cells involved in the specific immune response include various populations and subpopulations of T and B lymphocytes. Each individual B or T are highly specific and can only recognize one given antigen (Kuby, 1997). Once the cells of the specific immune system face an initial attack by an antigen, all subsequent responses to that same antigen occur more efficiently. This is due to a unique property of the specific immune system called a memory response.

The specific immune system is divided into two main subcategories- humoral and cell mediated immunity. B cells are involved in humoral immunity. Humoral immunity can be adoptively transferred from an immunized donor to a naive host. This transfer of humoral immunity is not dependent upon a transfer of the B cells themselves, rather on the transfer of the product of an activated B cell, which are the immunoglobulins (more

**Table 1: Features of Innate and Specific (Adaptive) Immunity**

<b>Feature</b>	<b>Innate</b>	<b>Specific (Adaptive)</b>
<b>Characteristics</b>		
Specificity for antigen	Relatively low	High
Diversity of Ag responses	Limited	Large
Improved response following 1 <sup>o</sup> Ag exposure	No	Yes
<b>Components</b>		
Physical and Chemical Barriers	Skin, mucosal epithelia, anti-microbial chemicals	Cutaneous and mucosal immune systems, secreted antibodies
Blood Proteins	Complement	Antibodies
Cells	Phagocytes (macrophages, neutrophils), Natural Killer cells	Lymphocytes- (populations and subpopulations of B and T cells)

commonly referred to as antibodies). The elimination of antigen is dependent upon recognition by and production of immunoglobulin (Ig) molecules. Antibodies are initially produced by the B cell as a membrane bound structure (IgM) that serves as the B cell's antigen receptor. Once the membrane bound immunoglobulin recognizes the antigen which it is programmed to eliminate, that B cell will differentiate into one of two types of progeny, a memory cell or a plasma cell. The memory cells will become inactive and serve as 'surveillance' cells, waiting for the pathogen that caused it's production to attack again (Kuby, 1997). The plasma cells, however, serve a much different function. The plasma cell is responsible for producing secreted antibody. The antibody which is secreted by the plasma cell will either be of the same isoform (IgM) or of a different isotype such as IgG, IgA, or IgE. Once the antibody is secreted, it will bind to the remaining free antigen causing various effector functions of the immune system to eliminate the antigen.

Cell mediated immunity involves the activation of T cells. An adoptive transfer of cell mediated immunity can only be successful if the transfer includes activated T cells (Abbas *et al.*, 1997). Like B cells, T cells, once activated will rapidly divide and produce either effector cells or memory cells. Unlike B cells, however, The T cells involved in the cell mediated immune response can themselves be divided into two main categories: T helper cells (TH) and T-cytolytic cells (CTLs). TH cells will not directly kill the antigen, rather they will stimulate other cells of the immune system to perform their functions. TH cells recognize foreign peptides presented to them on cell surfaces and secrete proteins which stimulate other cells, such as B cells, macrophages, neutrophils and other T cells. Without TH cells, all aspects of the immune response, including

innate immunity, would not be as efficient. Cytolytic T cells (CTLs) are the cells of the cellular immune system that will recognize and directly eliminate virally-infected or tumor cells. CTLs kill by the lysis, or breaking open, of the target cell's membrane. CTL killing is antigen specific and CTLs will only kill the target cells that present the antigenic peptide that the CTL is programmed to destroy. The CTL must be triggered by the recognition of the target cell/antigenic peptide before it will begin killing. Intimate contact between CTL and target cell is necessary for lysis to take place.

Unlike B cells, T cells cannot recognize free antigen and must recognize antigen in the context of an MHC molecule, or major histocompatibility complex molecule. The requirement that the T cell receptor (TCR) bind to antigen fragments in conjunction with self-MHC is called MHC restriction. MHC restriction of T cells takes place in the thymus during maturation. T cells migrate from the bone marrow, where they begin development, to the thymus where the T cells will complete their maturation and are prepared to enter the circulation. MHC molecules are present on all cells with the exception of sperm and oocytes. These MHC molecules are essentially recognition proteins which indicate to the body that the cell is a "self" cell. There are three classes of MHC molecules, MHC class I, MHC class II and MHC class III. MHC class I molecules are recognized by surface receptors on CTLs. MHC class II molecules are recognized by cell surface receptors on TH cells. Antigenic peptides are presented to T cells by antigen presenting cells (APCs). The antigenic peptide, or epitope, is presented to the T cell by the presenting cell's MHC complex. Once the T cell recognizes the antigen-MHC complex, the T cell will carry out its normal effector or cytotoxic function.

The step in T cell cytotoxicity is antigen recognition and the conjugation of target and CTL. Next, the cross linking of the TCR, which is bound to the MHC-antigen complex, and cell surface molecule CD3 causes the CTL to become activated. Internal signals are then delivered telling the cell to release the cytoplasmic granules (cytotoxins and pore-forming proteins such as perforin) responsible for lysis. The CTL is released from the target and the target cell dies by programmed cell death or apoptosis.

Natural killer (NK) cells, one of the main components in innate immunity, are similar to T cells in that they are involved in the cell-mediated immune response. Although there are some marked similarities, natural killer cells represent a population of lymphocytes that are morphologically and functionally different from the typical B and T cell (Suzuki *et al.*, 1992). NK cell activity was first described in the early 1970's. In one experiment conducted by Dr. R.B. Heberman and his colleagues (Heberman *et al.*, 1975), lymphoid cells from athymic mice were harvested and the level of cytotoxicity the cells expressed was assayed. The lysis of tumor cells and virally infected cells by such lymphoid cells prior to any type of immunization allowed for further investigation and description of NK cell ontogeny and function.

NK cells are found in animals as primitive as starfish and earthworms. This fact led scientists to believe that NK cells may have been the precursor to the evolved T and B lymphocytes (Robertson and Ritz, 1990). Like the B cells and T cells, NK cells begin their maturation in the bone marrow. NK cells usually complete their maturation in the bone marrow and don't undergo MHC restriction. Natural killer cells comprise approximately ten to fifteen percent of all peripheral blood lymphocytes and about 3-4% of all lymphocytes in the spleen (Sivakumar *et al.*, 1998). The spleen is the

richest source for functional and mature lymphocytes in both humans and mice. Unlike T cells, which are found in the white pulp of the spleen, the NK cells are found in the red pulp. NK cells have also been found in the liver, interstitial compartments of the lung and intestinal mucosal (Sanchez *et al.*, 1993).

The MHC molecule plays opposite roles in NK and T cell function. T cells are activated by the presentation of an antigen by a self-MHC molecule. This allows the T cell to discern which self cells have been infected with foreign antigen or transformed into a tumor cell. The T cell receptor itself has a specific region that recognizes the MHC as well as a specific region that recognizes the antigenic particle, or epitope. The TCR's recognition of the MHC molecule in conjunction with the epitope will activate the T cell to lyse the target cell membrane or to produce signals to induce activation and proliferation of other lymphocytes.

NK cells also have MHC specific receptors on their surface. However, when these receptors come into contact with an MHC molecule, they will inhibit the NK cell's cytotoxic activity (Karre, 1995; Chambers *et al.*, 1998; A. Moretta *et al.*, 1999.) The NK cell has a variety of such receptors on its surface which recognize specific MHC class I molecules (Table 2). In humans it is the p58 family of receptors, but in murine NK cells the Ly-49 family of receptors are responsible for recognizing MHC class I as self (Ortaldo *et al.*, 1999; Lian *et al.*, 1999). There is also an array of ligands and receptors which are involved in the inactivation and activation of NK cells (Kubota, 1997). NK cells require the binding of the target cell's class I molecules to the NK cell's Ly-49 receptor in order for an inhibitory signal to be sent to the NK cell.

**Table 2: Natural Killer Cell Receptors/Markers**

Receptor Function	Human NK cell Receptor	Mouse NK Cell Receptor
Recognize MHC	P58 family	Ly-49 family
ADCC	CD16	CD16
Adhesion to target cell	CD56	NK 1.1

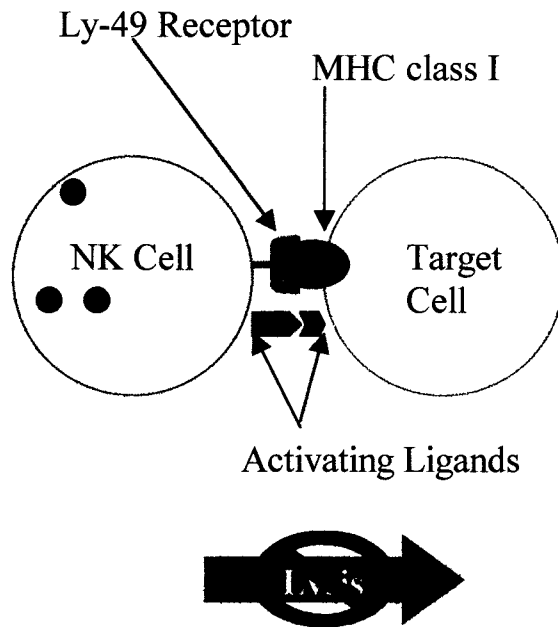
The NK cells are able to recognize and eradicate foreign cells or virally infected cells which have down regulated the host cell's MHC class I expression on its surface (Correa *et al.*, 1994). Some virally infected or tumor cells do not cause down regulation of MHC molecule production and antigenic peptides are still presented on the surface of the infected or mutated cell. T cells are able to detect and destroy these cells. Other virally infected cells and tumor cells down regulate the production of MHC class I on cell surfaces. NK cells will kill these virally infected and tumor cells which do not exhibit high frequencies of MHC class I on their surface. This characteristic of NK cells give the immune system an advantage in detecting and killing virally infected cells and tumor cells which were able to bypass detection by T cells.

Like T cells, natural killer cells kill their target cells by lysis of the target cell's membrane. Unlike T cells, NK cells are not antigen specific. They lack T cell receptors and/or immunoglobulins on their surface. Because they are not antigen specific, and their ability to lyse target cells is also not dependent upon the recognition of an MHC complex, NK cells can serve as one of the body's first lines of defense against infection. Natural Killer cells will not just kill any cell, however. NK cells are programmed to kill only certain virally infected cells and certain tumor cell lines which downregulate MHC class one on their surface. NK cells will not kill uninfected cells. NK cells utilize a similar mechanism in the lysing of target cells as CTLs. Once NK cells bind to a target cell with absent or depleted MHC molecules on it's surface, signals cause the secretion of cytotoxic granules such as perforin and granzymes (Figure 1). Once the cytotoxic granules are released and cross over the target cell membrane, the target

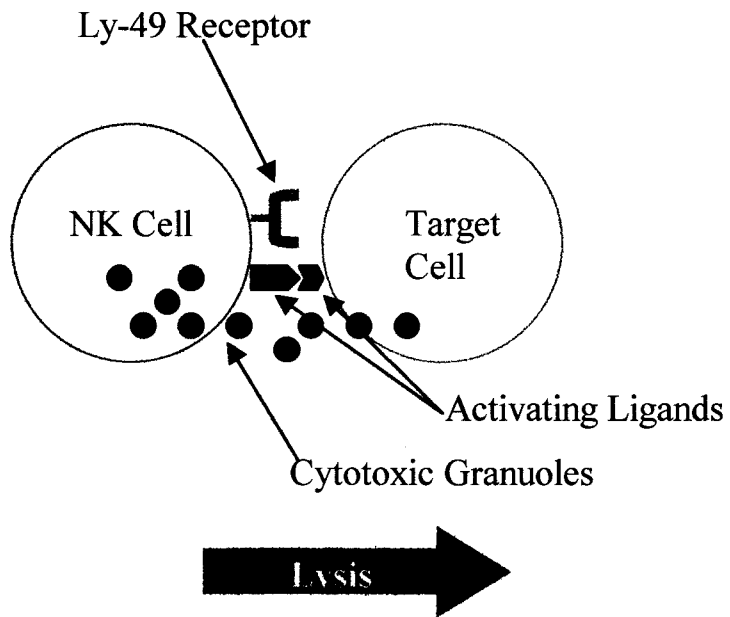


**Figure 1. A) Inhibition of Cell Death:** MHC-class I recognition by the Ly-49 receptor of the mouse NK cell inhibits the release of cytotoxic granules. Because these granules are not released, the cell will not die.

**B) Induction of Cell Death:** The downregulation or absence of MHC class I due to virus infection or tumor cells results in a lack of inhibition of NK cell release of cytotoxic granules and subsequent death of the target cell.



A. Inhibition of Cell Death



B. Induction of Cell Death

cell dies by programmed cell death (apoptosis), or lysis (membrane damage).

Another marked difference between T cells and NK cells is the different cell surface markers displayed by each type of cell. Each functionally distinct population of lymphocytes expresses specific membrane proteins called cluster of differentiation (CD) molecules. These CD molecules can serve as phenotypic markers as well as adhesion points for each kind of lymphocyte. For example, all T cells, both effector and cytotoxic, have a CD3 molecule attached to its T cell receptor (TCR). NK cells can be recognized by the presence of distinctive surface molecules such as CD16, CD56 (humans only), NK 1.1 and DX5 (mice only) markers accompanied by the absence of CD3 (exclusive to T cells) or Ig molecules (unique to B cells). There are two CD markers, CD16 and CD56, which are found in high frequencies on the surface of human NK cells (L. Moretta *et al.*, 1992). However, CD16 and CD56 are also found in low frequencies on certain subpopulations of T cells which aren't MHC restricted, and CD16 can be found on various types of phagocytic cells. CD 16 is responsible for inducing antibody dependent cell cytotoxicity (ADCC), a second mechanism of killing in addition to natural killing. CD16 is a low affinity Fc- $\gamma$  receptor on the surface and within the cytoplasmic membrane of the NK cell (and other phagocytic cells) which recognizes the bound antibody IgG. Target cells which display aggregations of IgG on its surface will successfully bind to the NK cell's CD16 receptor. Once the CD16 itself aggregates into a cluster, cytokines such as tumor necrosis factor (TNF) and interferon- $\gamma$  are released as well as the cytotoxic granules. Therefore, the lysis of the target cell as well as the induction of the immune response due to the release of cytokines may be accomplished by ADCC. CD 16 is found in

high frequencies on both human and murine NK cell surfaces (Robertson and Ritz, 1990).

Cell surface molecule, CD56, is found on human NK cells and antibodies directed against it have provided an excellent mechanism for identifying NK cells in humans. Unfortunately, CD56, also called the neural cell adhesion molecule (NCAM), is not found on mouse NK cells. A similar marker, NK 1.1 is responsible for performing the same function in mice as CD56 does in humans by mediating adhesion to target cells. The first murine monoclonal antibody which identified cell surface molecules of NK cells binds to NK 1.1 (Sivikumar *et al.*, 1998). Unfortunately, not all strains of mice have NK cells that express NK 1.1 on their surface. However, in the strains of mice that do express NK1.1, NK cells also express a surface antigen found linked to NK1.1. This surface antigen is known as DX5 in murine natural killer cells. DX5 is expressed on the surface of virtually all NK cells but its function is unknown. Antibodies against DX5 can be used to identify NK cells in strains of mice, such as the Swiss Webster mouse strain that do not have the cell surface antigen NK1.1 on NK cell surfaces.

A pure NK cell population is needed to study NK cell function because NK cells are greatly outnumbered by T cells and it is difficult to separate them out of mixed lymphocyte cultures. NK cells are also difficult to isolate because NK cells and T cells express many of the same cell surface antigens and perform similar functions. Another difficulty in the creation of a pure NK cell preparation lies in the fact that there have been some “NK-like” T cells (NK-T cells), isolated from NK clones, that exhibit properties of both NK cells and T cells (Versteeg, 1992).

Because these NK-T cells do exist, it is not only important to differentiate T cells from NK cells, it is also important to differentiate NK-T

cells from NK and T cells. NK cells, T cells and NK-T cells are similar in size and density. NK cells, T cells and NK-T cells also release similar cytokines. All kill in a similar way, releasing some of the same cytotoxic factors, such as perforins and granzymes (Archa-Orbea *et al.*, 1983). However, unlike T cells or NK cells, NK-T cells express both CD 6 (human), or NK 1.1 (murine) as well as CD3 and T cell receptors on their surface and cause spontaneous lysis of virus-infected or tumor cells which are not MHC restricted (Hammond *et al.*, 1999). This small population of lymphocytes represents those NK-like cells which are specific for antigens presented in conjunction with MHC I and MHC II (Slifka M.K. *et al.*, 2000). In this study of NK cells, T cells and NK-T cells will have to be clearly identified and eliminated from the cell population studied.

In order to study pure NK cell populations, our lab has been attempting to develop a NK cell hybridoma. The creation of hybridoma cells involves the fusion of lymphocytes to myeloma cells (Morgan and Darling, 1993). A normal lymphoid cell will not survive long nor will it have the ability to rapidly proliferate *in vitro*. A myeloma cell, which is a cancerous lymphoid cell, is able to sustain itself *in vitro* for extended periods of time (McKinney and Belfort, 1996). The creation of a NK cell hybridoma would provide a homogenous pool of rapidly growing cells with NK cell properties and functions that will grow indefinitely in culture. This provides an unlimited pool of NK cells to study. NK cell clones have previously been created, but these cells have not survived for long periods of time in culture (Sivakumar *et al.*, 1998).

In order to select for hybridomas, the fused cells must be initially grown in HAT media. HAT media creates an environment in which only properly fused cells will survive. Cells which remain un-fused along with

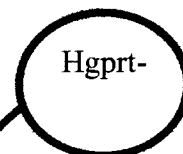
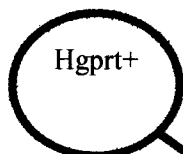
cells that fuse improperly, such as a myeloma-myeloma fusion or a lymphocyte-lymphocyte fusion, will not survive. The lymphocytes which fused to each other cannot be sustained for long and will die after a short time *in vitro*. The myeloma cells which fused to each other will die due to a lack of a specific enzyme which would allow other cells such as lymphocytes to thrive in HAT media. There are two possible pathways that can be used by cells to synthesize nucleotides: the salvage pathway and the *de novo* pathway (Figure 2). The myeloma cells used for fusion are deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Abbas *et al.*, 1997). This enzyme is essential to the salvage pathway of nucleotide synthesis. HAT media is composed of hypoxanthine, aminopterin and thymidine. The aminopterin blocks the utilization of the *de novo* pathway of nucleotide synthesis, while the thymidine and the hypoxanthine induces nucleotide production via the salvage pathway. Inhibition of *de novo* DNA synthesis by aminopterin kills the improperly fused myeloma cells as they are not able to use the salvage pathway because of their HGPRT deficiency. The only cells that remain after HAT selection will be the hybrid cells.

Previous studies in this lab (unpublished observations), attempted to identify NK cell hybridomas using the biotin-conjugated rat anti-mouse Pan-NK (anti-DX5) cell monoclonal antibody. However, because DX5 is a low affinity binding antibody, histochemical analysis of NK cell hybridomas was not very efficient. Flow cytometry was next investigated as a more efficient method of NK cell hybridoma detection. A biotinylated primary antibody was used that selectively bound to the DX5 antigen. A secondary, fluorescently-labeled antibody was then allowed to bind to the primary antibody. The fluorescently labeled antibodies were then detected by the use

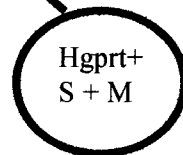
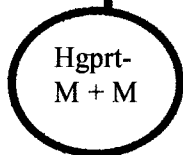
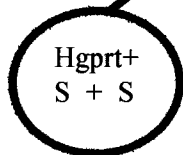
**Figure 2. HAT Selection:** Growth of newly fused cells in HAT media will allow for the selection of properly fused cells. Only those fused cells which are HGPRT<sup>+</sup> will survive in HAT media. Although spleen cells which fuse to spleen cells survive the HAT media because they have the hgprt enzyme, they soon die because they cannot survive long term in culture.

Spleen Cell (S)

Myeloma Cell (M)



FUSION



Survives:

Death

Survives:

Not long term

Colonies Form



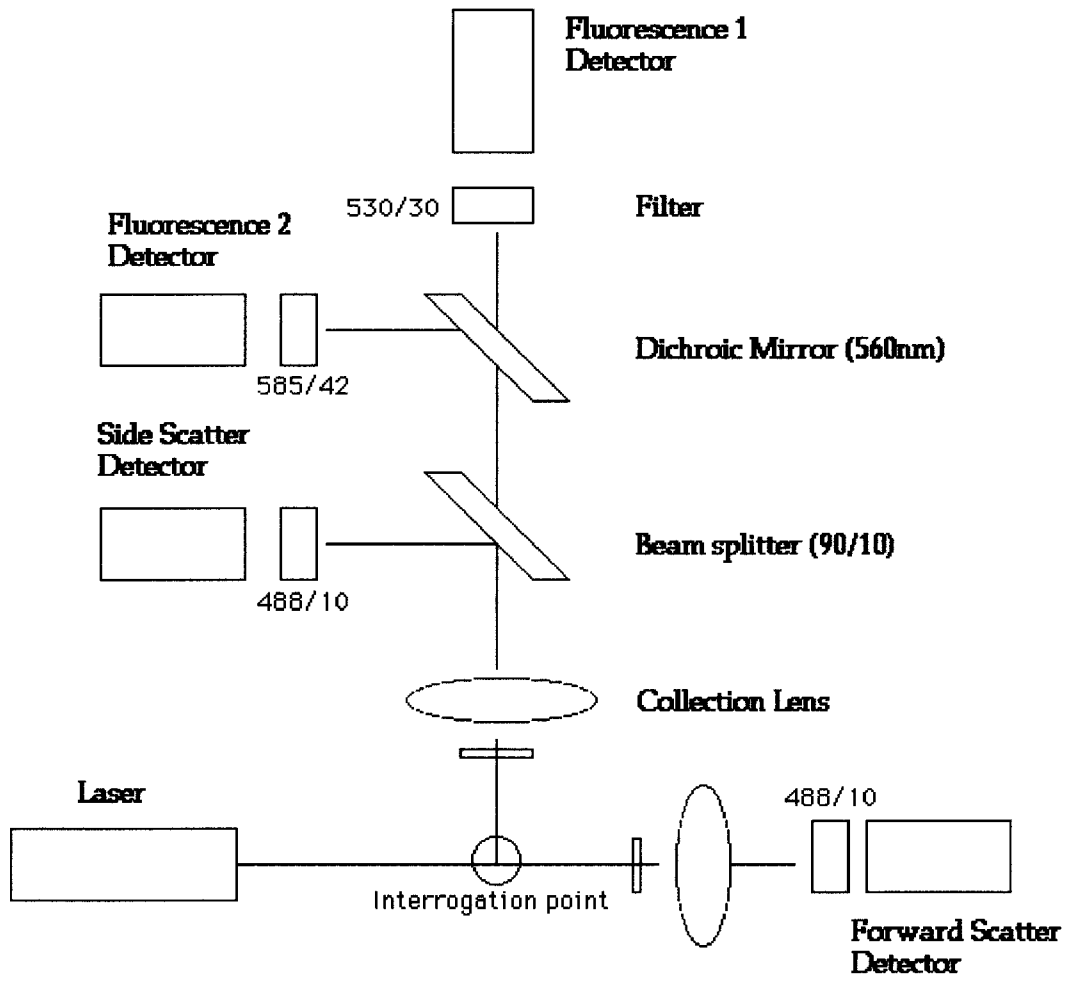
of flow cytometry. Flow cytometry allows for the study and identification of specific cell types in a heterogeneous population. Flow cytometric analysis is a useful method to analyze the morphological and chemical characteristics of cells. Fluorescently labeled cells pass in a fluid stream one by one through a flow chamber. The cells, suspended in liquid, come into contact with a laser beam at the interrogation point (Figure 3). Depending upon its size and granularity, a cell, when in contact with the laser beam will diffract, or scatter the light. The scattered light is then converted into electrical impulses by a photodetector lens. The forward scatter detector (FSC) indicates the size of the cell which is passing through the interrogation point. The side scatter (SSC) detector measures the cell's granularity (Givan, 1992).

The laser beam also serves a secondary function. As the laser hits the cell's surface it activates the fluorescent label (FITC or PE) which is conjugated to a primary antibody. The now activated cell will emit energy in the form of light and a fluorescence detector measures the emission of this light. There are two fluorescence detectors, a FITC fluorescence detector and a PE fluorescence detector.

In these studies, cell pools which stained positive for DX5 will be identified by flow cytometry and then be subcloned in a 96 well plate allowing for some wells to contain one clone. Those cells containing one or two clones were then stained and tested for the DX5 marker in an attempt to detect NK cell hybridoma clones. Cells which stained positive for DX5 were also tested for the absence of the T cell marker CD3, to confirm that the clones were not contaminated with T cells. Desirable clones should be DX5<sup>+</sup> and CD3<sup>-</sup> which includes NK cell populations but excludes T cell and NK-T cell populations.

**Figure 3. Diagrammatic Representation of a Typical Flow Cytometer:**

The cell, in suspension, comes into contact with the laser beam at the interrogation point. Depending on the size and granularity of the cell, the cell will scatter the light forward or to the side where it is detected as an electrical impulse by one of two detectors. Also, the laser activates the fluorescent label which is also identified by one of two detectors.

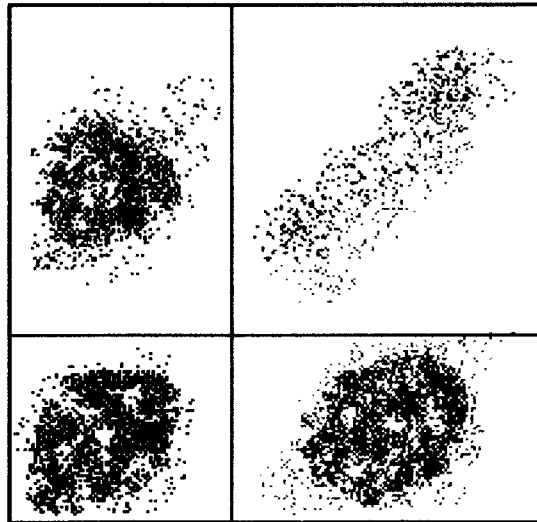


Flow cytometric data is typically analyzed by electronic gating. Once the selected number of cells has been analyzed by the flow cytometer, the data appears on the computer screen as a logarithmic dot plot. The dot plot can then be separated into sections or “gates.” The cells that lie within the gate or gates are the ones that will be numerically analyzed or included in a histogram (Martin and Buevery, 1993). The dot plot can be separated into four quadrants (Figure 4). The upper left quadrant will represent the cells detected that were found to be emitting energy at the same wavelength as PE (Figure 5). These cells were labeled with anti-mouse CD3-PE indicating the presence of the CD3 molecule. The lower left quadrant will represent the cells detected that were found to be emitting energy at the same wavelength as FITC. This indicates that the cell has an anti DX5-FITC antibody bound to its surface. The upper right quadrant will represent the cells detected that were found to be emitting energy at the same wavelength as FITC and PE simultaneously. This indicates that the cell either has both FITC and PE bound to its surface, or that the cell itself has fluorescent properties. The lower left quadrant represents cells that have no bound fluorescent antibodies (Mandy, 1995).

By using a standard hybridoma fusion protocol, fluorescently-labeled antibody staining and flow cytometry, the primary goal of this laboratory was to isolate and clone a natural killer cell hybridoma. A NK cell hybridoma clone pool could then be used as a continual resource of NK cell hybridomas for future studies in the laboratory.

**Figure 4. Quadrant Fluorescence:** Gated dot plot analysis of flow cytometric data can be separated into four quadrants: Q-UL: represents cells fluorescing at the wavelength of PE, Q-LL: represents unlabeled or non-fluorescing cells, Q-UR: represents cells which fluoresce at the wavelengths of both FITC and PE, Q-LR: represents cells fluorescing at the wavelength of FITC.

(Q-UL)

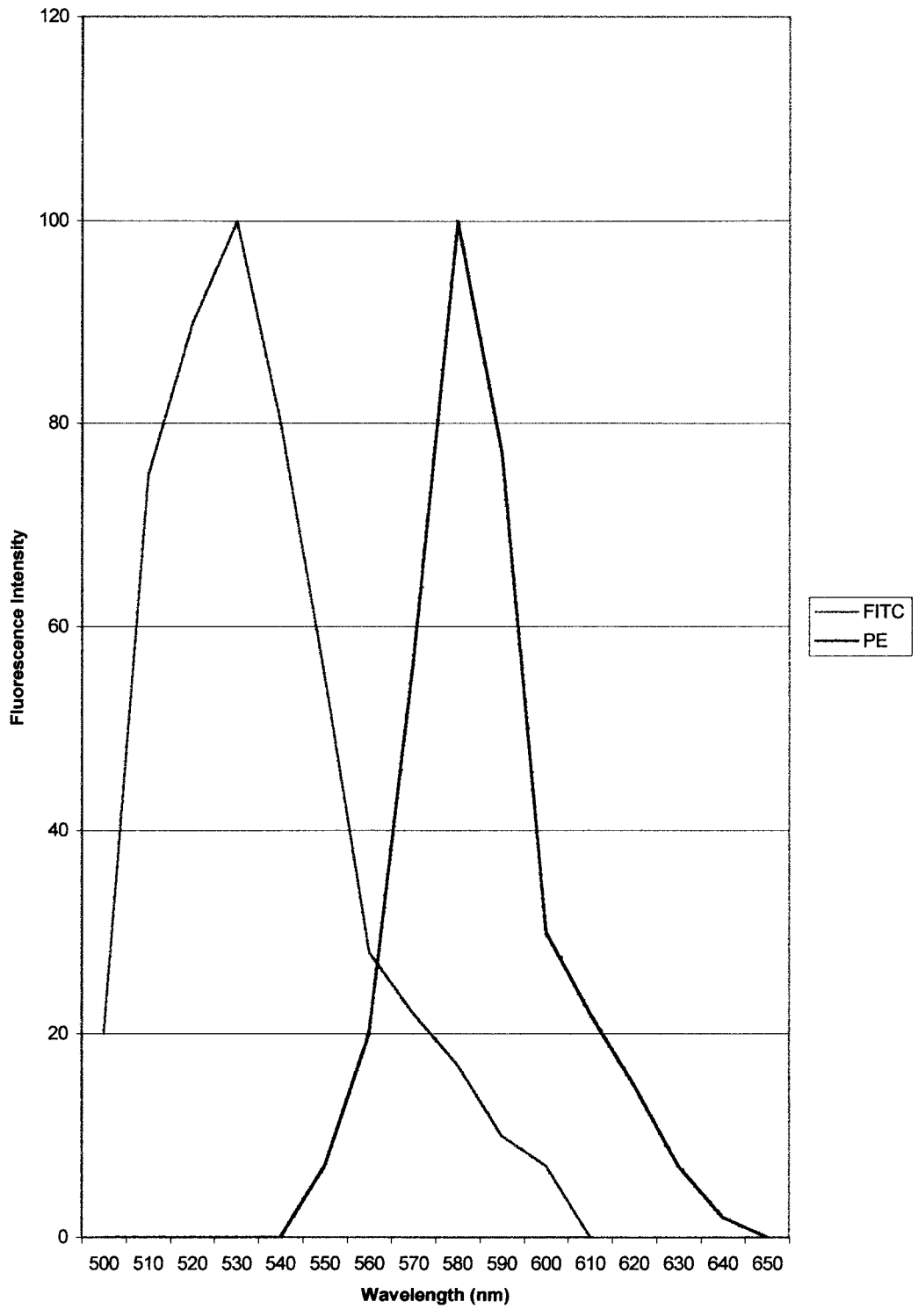


(Q-UR)

(Q-LL)

(Q-LR)

**Figure 5. Spectral Characteristics of Selected Dyes:** The x-axis represents the wavelength of excitation emitted by the fluorescent dyes. The y-axis represents the fluorescence intensity, or the amount of fluorescence detected at various wavelengths.





## II. MATERIALS

Sodium Bicarbonate (tissue culture tested), polyethylene glycol (PEG), Tris[hydroxymethyl]aminomethane (tris base), Tris-ammonium chloride, monobasic sodium phosphate, monobasic potassium phosphate, sodium azide, hypoxanthine-aminopterin-thymidine (HAT), n-octyl-B-D-glucopyranoside (OG), paraformaldehyde and trypan blue were purchased from SIGMA Chemical Company (St. Louis, MO). Fetal calf serum (FCS), 100X L-glutamine, 100X sodium pyruvate, Hepes buffer, RPMI- 1640, and minimal essential media with Earls Salts were purchased from GIBCO BRL (Grand Island, NY). Purified rat anti-mouse CD16/CD32 (Fc $\gamma$ III/II Receptor) monoclonal antibody (Fc-block), biotin-conjugated rat anti-mouse Pan-NK cell monoclonal antibody, strepavidin-fluorescein isothiocyanate (FITC) conjugate, R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD3 antibody were purchased from PharMingen (San Diego, CA). Cytoperm/Ctyofix™ was purchased from Becton Dickinson (San Jose, California). P3X63-Ag8.653 (non-secreting, mouse myeloma) cells and MRC-5 (human fibroblast) cells were purchased from American Type Culture Collection (ATCC, Rockville, MD).

### **III. METHODS**

#### **A. SOLUTIONS:**

##### **Supplemented RPMI-1640 media:**

The RPMI-1640 media (90mls) was supplemented with sodium bicarbonate (1.5g/100ml), heat inactivated FCS (10%), 29.2% L-glutamine, 0.25% glucose solution (1ml), Hepes Buffer (1ml) and 100X sodium pyruvate (1ml). The pH of the solution was adjusted to 7.2-7.4 with 0.1N HCL or 0.1N NaOH. (This media was also used to maintain the P3X and hybridoma cells).

##### **Supplemented Earle's Minimal Essential Media (MEM):**

The Earle's MEM (90mls) was supplemented with sodium bicarbonate (1.5g/100ml), heat inactivated FCS (10%), 100X sodium pyruvate (1ml) and 29.2% L-glutamine (1ml). The pH of the media was adjusted to 7.2-7.4 with 0.1N HCL or 0.1N NaOH.

##### **50% PEG:**

Approximately 20-50 grams of PEG is melted in a 100ml glass reagent bottle and steam autoclaved for 20 min at 121°C-132°C. As it cooled, but before it solidified, an equal volume of RPMI-1640 media (unsupplemented) was added.

##### **10X Phosphate Buffered Saline (PBS):**

Monobasic sodium phosphate (hydrous- 7.95g) was combined with 1.44g of monobasic potassium phosphate and 800ml of MilliQ water. Sodium chloride (90g) was then added and the pH of the solution was adjusted to 7.4

with 0.1N HCL or 0.1N NaOH. The solution was brought up to 1L with MilliQ water and stored at room temperature.

**Paraformaldehyde:**

PBS (10ml of 10X) and 10mg of paraformaldehyde were added to 90ml of MilliQ water. The solution was heated to 56°C in a water bath and allowed to cool to room temperature. Once cooled, the pH of the solution was adjusted to 7.4 with 0.1N HCL or 0.1N NaOH. The 0.01% paraformaldehyde can be stored for two weeks at 4°C.

**PBS-Azide:**

10X PBS (10 ml), milliQ water (90ml) and Sodium Azide (0.1g) were combined to make PBS-Azide. This solution was stored for up to one week at 4°C.

**n-Octyl-B-D-Glucopyranoside (OG):**

n-Octyl-B-D-Glucopyranoside (OG) (0.06g) was dissolved in 9ml of milliQ water and 1ml of 10X PBS was added.

**B. CELL CULTURE:**

P3X63-Ag8.653 were maintained in RPMI-1640 supplemented media. The P3X were grown in a flat-bottomed flask in a 37°C, 5% carbon dioxide enhanced incubator. The MRC-5 were kept in a 37°C, 5% carbon dioxide enhanced incubator and maintained with supplemented Earle's minimal essential media (90ml)

### **C. CELL FUSION (HYBRIDOMA) PROTOCOL**

A Swiss-Webster mouse was sacrificed by carbon dioxide inhalation. To avoid any unnecessary contamination the dissection was done under a sterile hood and the mouse's fur was saturated with ethanol. The mouse's spleen was removed aseptically and placed in cold PBS at physiological pH. The spleen was then transferred into serum-free RPMI-1640 into a well of a 24 well plate. A sterile plunger was then used to disrupt the spleen in order allow for the spleen cells to be released. The remaining clumps were allowed to settle for five minutes. The supernatant was then collected and centrifuged at 400 x g for eight minutes. The pellet was then resuspended in Tris-ammonium chloride, layered over fetal calf serum, and centrifuged at 300 x g for ten minutes in order to remove the dead cells. The cells which remained atop the fetal calf serum (the lymphocytes) were then collected and counted. The spleen cells were then diluted to  $1 \times 10^8$  cells/ml with supplemented RPMI-1640.

The P3X mouse myeloma cells which were in the log phase of growth were collected and counted. The P3X cells were then diluted to  $2 \times 10^7$  cells/ml with supplemented RPMI-1640. The P3X and spleen cells were then combined and centrifuged at 200 x g for eight minutes. The supernatant was aspirated and 50% PEG/RPMI (1ml) was added drop-wise to the pellet for one minute while gently shaking the tube. Warm (37°C), serum-free RPMI (1 ml) was added to the suspension drop-wise for one minute while gently shaking the tube. The cells were then centrifuged at 200 x g for eight minutes. The supernatant was removed and the pellet was resuspended in serum-free supplemented RPMI-1640 (24mls). This suspension of cells was then placed in 1ml aliquots into twenty-four well

plates. The wells of the twenty-four well plates had been previously seeded with  $2 \times 10^5$  cells/ml MRC-5 feeder cells which were actively growing and adhering to the bottom of the wells. The cells were maintained by incubation in a carbon dioxide supplemented incubator.

#### **D. HAT (HYPOXANTHINE-AMINOPTERIN-THYMIDINE) SELECTION**

After allowing the cells to proliferate for 1 day in the presence of supplemented RPMI media, the hybridomas were ready for HAT selection. Fifty percent 2X HAT was added to the recipe for supplemented RPMI media. For two weeks, 1ml of this media was added to each of the 24 wells three times a week. At the end of the two weeks, the cells were then fed with the supplemented RPMI containing HT for one week. During the subsequent weeks, the hybridoma cells were fed with supplemented RPMI. The hybridomas were allowed to proliferate until they were 2/3 confluent. The cells were then cultured in 6 well plates until there were enough cells for antibody staining.

#### **E. ANTIBODY STAINING PROTOCOL**

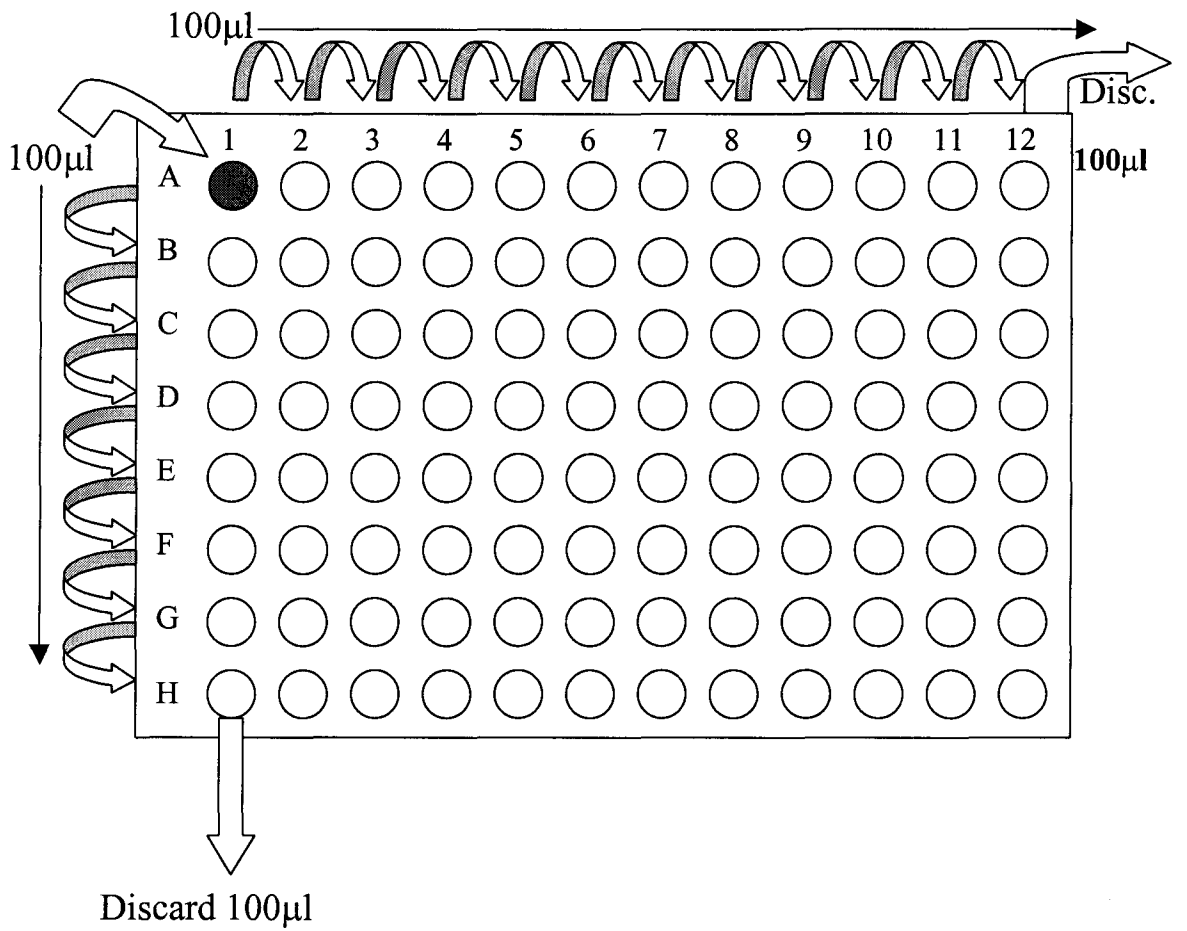
The cells from each well were counted with a hemacytometer. One milliliter of each cell pool was then diluted to  $1 \times 10^7$  cells/ml. The cells (1ml in each tube) were then washed with 2mls PBS Azide three times. After the third wash, the cells were resuspended in 2mls of PBS azide. The cells were then suspended in 1ml of media and 100 $\mu$ l of the cells, leaving a final concentration of  $1 \times 10^6$  cells/ml, were placed in each of two

polyethylene tubes labeled accordingly for each cell pool. Fc block (5 $\mu$ l), diluted 1:10 in PBS azide was then added to each tube. The cells were incubated in the dark at 4°C for approximately four minutes. The primary antibody, Pan-NK (5 $\mu$ l), was then added to half of the tubes for each cell pool and all cells were incubated for thirty minutes in the dark at 4°C. The cells were then centrifuged for five minutes at 400 x g. The pellet was then washed three times in 2mls wash buffer and centrifuged at 400 x g for eight minutes. Secondary antibody, strepavidin-FITC, in 100 $\mu$ l of wash buffer was then added to all of the tubes. At this point, half of the tubes contained both primary and secondary antibody, and the other half contained secondary antibody and was used as a negative control. The cells were incubated in the dark at 4°C for thirty minutes. The suspension was then centrifuged for eight minutes at 400 x g. The cells were washed with 2mls PBS azide three times. After the final wash, the pellet was resuspended in 500 $\mu$ l of wash buffer.

## **F. SUBCLONING**

Cell pools that stained highly positive for the NK cell surface antigen DX5 were chosen to be subcloned (Figure 6). 96 well plates were pre-seeded with  $2 \times 10^5$  cells/ml of MRC-5 feeder cells per well. The MRC-5 cells were allowed to incubate overnight. The supernatant from each of the wells was aspirated off. 100 $\mu$ l of supplemented RPMI media was added to each well. Cells from mixed lymphocyte hybridoma cell pool (100 $\mu$ l)

**FIGURE 6. Serial Dilution of Cell Pool C6:** Cells from cell pool C6 (100 $\mu$ l) were placed in well A1. The cells were then serially diluted by placing 100 $\mu$ l of cells from well A1 to well B1 and continue this procedure down the plate until the last well H1. Cells from H1 (100 $\mu$ l) were then discarded. The cells were then serially diluted with a multi-channel pipette by placing 100 $\mu$ l of cells from the first row (A1-H1) into the next row (A2-C2). This was continued across the plate until the final row (A12-C12), and 100 $\mu$ l of cells from the last row were discarded.





were added to well A1. The cells were then serially diluted down the plate from well A1 to well H1 with the dilution from the final well, H1, being discarded. Supplemented RPMI media (100 $\mu$ l) was added to each well. The cells were then serially diluted across the plate from wells A1-H1 to A12-H12, with the dilution from the final 8 wells (well A12-H12) being discarded. After four days of incubation, the subcloned cells were viewed under a light microscope. Those wells containing only one cell clone were marked. The cells were then fed with 100 $\mu$ l of RPMI and allowed to grow until confluent and then retested for cell surface antigens by antibody staining.

## **G. QUENCHING OF AUTOFLUORESCENCE USING TRYPAN BLUE**

Two methods were used in to fix and permeabilize the cell membrane before trypan blue treatment could occur. The first method included the use of paraformaldehyde and n-Octyl-B-D-Glucopyranoside (OG). Hybridomas from cell pool C6 ( $1 \times 10^6$  cells/ml) were placed into tubes and washed two times for 5 minutes at 400 x g. The pellets were resuspended in 200 $\mu$ l 0.1% paraformaldehyde solution (the fixing agent). After being fixed for five minutes at room temperature, the cells were washed once with 2ml 1X PBS. The pellet was resuspended in 0.6% n-Octyl-B-D-Glucopyranoside (OG) in order to permeabilize the cell membrane. Various concentrations of trypan blue ranging from .01 $\mu$ g/ml to 2 $\mu$ g/ml were then prepared and added to one of each of the tubes. The cells were allowed to sit for ten minutes at room temperature to allow the dye to take effect. After the time elapsed, the cells were washed twice and centrifuged at 400 x g for

8 minutes. The pellet was resuspended in 500µl cold PBS and analyzed by flow cytometry analysis.

The second method for quenching autofluorescence used Cytofix/Cytoperm™ solution followed by trypan blue. Hybridomas from cell pool C6 ( $1 \times 10^6$  cells/ml) were placed into tubes and washed two times for 5 minutes at 400 x g. While vortexing the pellet gently, 250µl of the Cytofix/Cytoperm™ solution was added to each tube. The suspension was allowed to sit at 4°C for 20 minutes. The cells were then washed two times in the wash solution (Perm Wash) provided in the Cytofix/Cytoperm™ kit (1ml of a 1X solution). Various concentrations of Trypan Blue ranging from 0.01µg/ml to 10µg/ml were then prepared and added to one of each of the tubes. The tubes were allowed to sit for 30 minutes at 4°C and then washed 2 times in 1 ml of 1X Perm Wash solution. The pellet was resuspended in 500µl cold PBS and analyzed by flow cytometry.

## **H. FLOW CYTOMETRY ANALYSIS:**

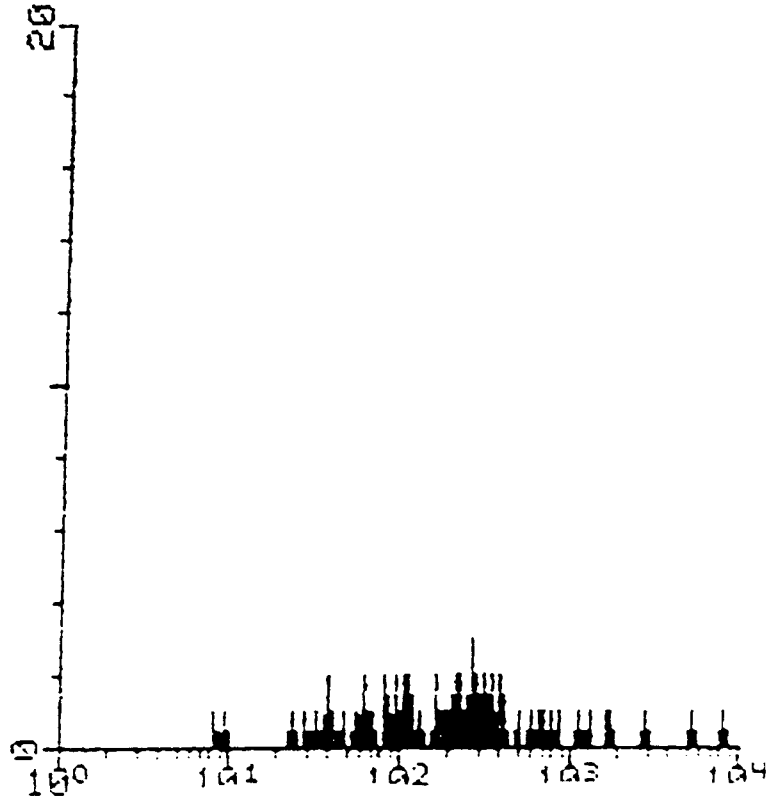
The cells were analyzed by flow cytometry using a Beckton Dickson FACScan at St. Elizabeth's Hospital. Cells are analyzed one by one as they pass singularly through a laser beam. The results were analyzed by electronic gating and statistical analysis. The gated area of the dot plot is the selected area or area of analysis (see Figure 4). The detected cells that lie within the gate or gates are the ones which will be statistically analyzed or included in a histogram. When the cell and laser beam interact it is referred to as an event. Each analysis included 5,000 events (cells).

For the first portion of our experiment, Lysis II software was used to analyze data from the experiments. Once the dot plots were gated, the area

selected was then represented by a histogram. The histogram shows the mean fluorescence of the cells included in the gated area of the dot plot (Figure 7). Midway through our experimental procedures and data collection new software, Cell Quest, was implemented. In order to make sure that any background noise or error was not coming from the flow cytometer itself, or to rule out possibilities of error due to mistakes in the usage of the new software, control beads were analyzed. The control beads were labeled with PE and FITC (positive control)(Figure 8) and some were left unlabeled (negative control)(Figure 9). The beads were detected and analyzed properly by the flow cytometer and the new software.

**Figure 7. Histogram Illustrating the Mean FITC Fluorescence of Included in Gated Area of Dot Plot:** The logarithmic histogram shows the mean fluorescence intensity emitted by the cells analyzed in selected gate (see Fig 4) of the dot plot being analyzed. The histogram statistics, located below the histogram, will show the numerical mean fluorescence.

/4/NDA012



/4/NDA012

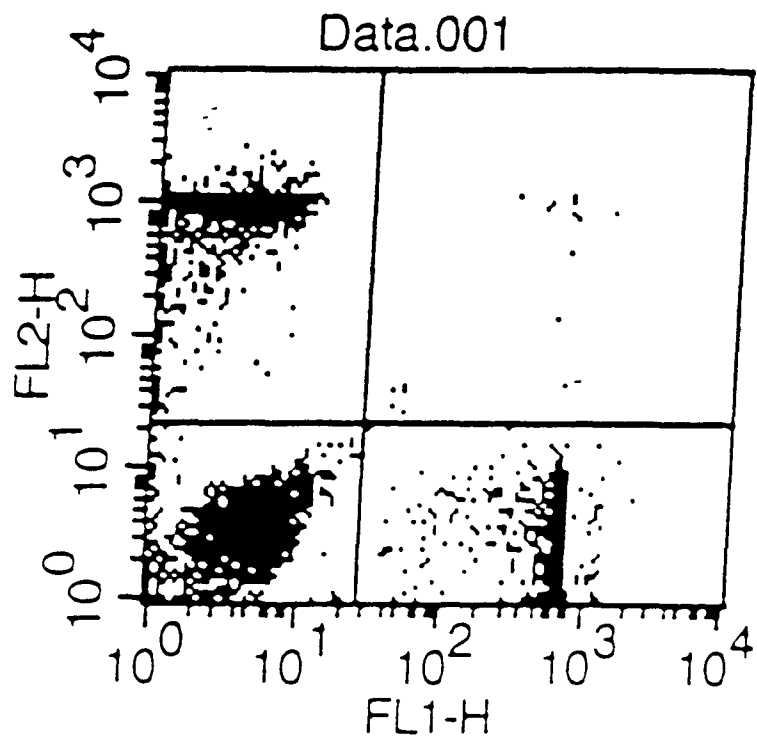
--- Arithmetic Histogram Statistics for /4/NDA012 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H Gate G2= R2

M	Left,Right	Events	%	Peak	PKCh1	Mean	Median	SD	CU %
0	1.00,	9646	68	100.00	3	264.16	522.96	212.88	1190.85 >100.1

**Figure 8. Dot Plot Analysis of Positive Control Beads:** The x-axis, FL1-H, represents the fluorescence intensity emitted by beads labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by beads labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



Quadrant Statistics

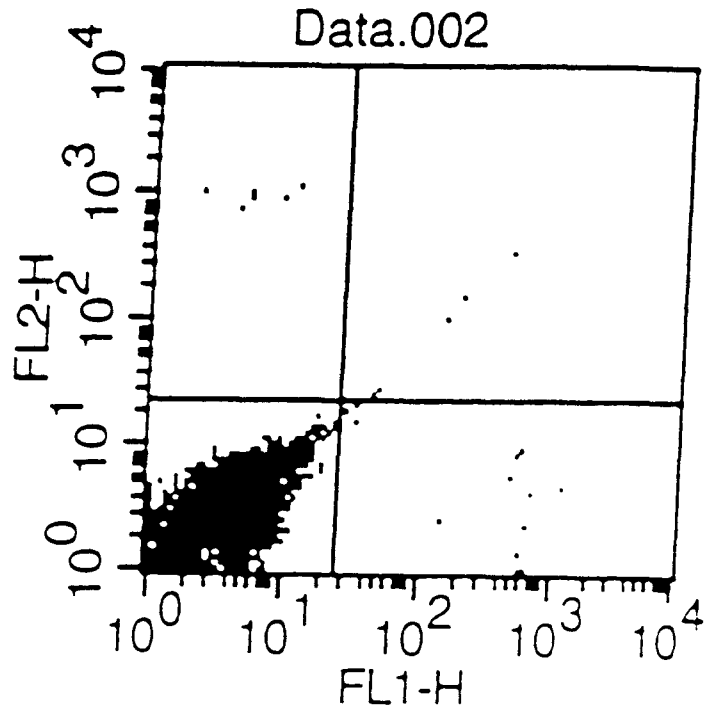
File: Data.001  
 Sample ID: ndd  
 Tube:  
 Acquisition Date: 31-May-00  
 Gated Events: 5000  
 X Parameter: FL1-H (Log)  
 Quad Location: 26, 22

Log Data Units: Linear Values  
 Patient ID: ndd  
 Panel:  
 Gate: No Gate  
 Total Events: 5000  
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1928	38.56	38.56	3.86	3.34	950.94	870.16
UR	18	0.36	0.36	475.90	295.45	599.83	279.37
LL	2074	41.48	41.48	5.35	4.58	3.95	3.49
LR	980	19.60	19.60	604.87	551.55	3.28	2.54

**Figure 9. Dot Plot Analysis of Positive Negative Control Beads:** The x-axis, FL1-H, represents the fluorescence intensity emitted by beads labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by beads labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.





Quadrant Statistics

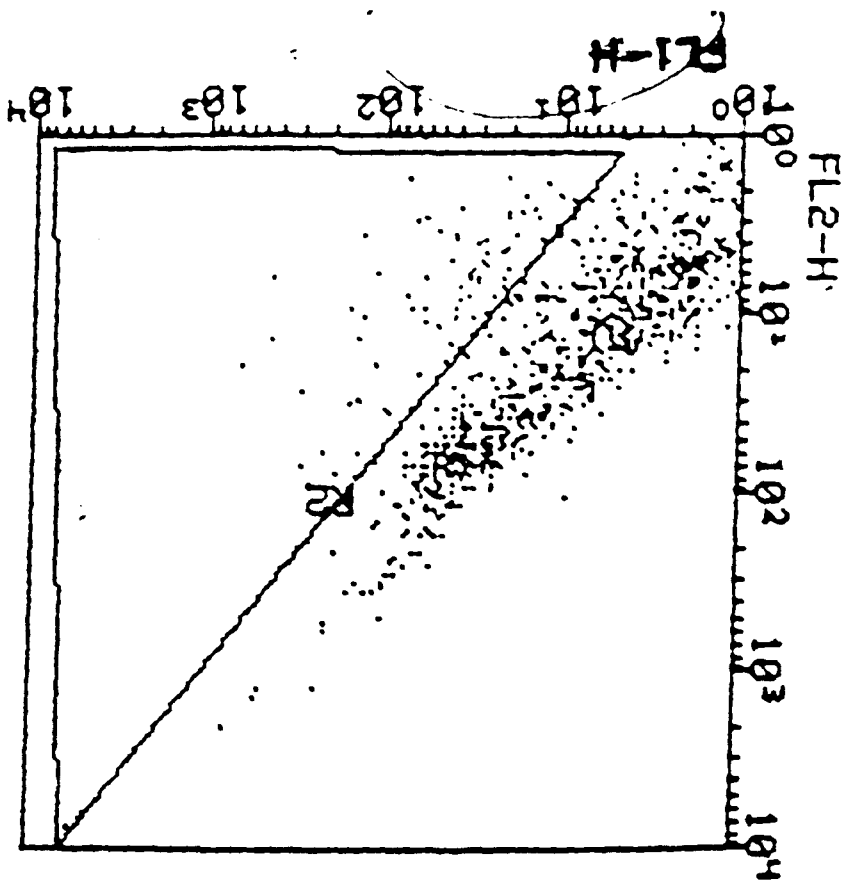
File: Data.002	Log Data Units: Linear Values
Sample ID: ndd	Patient ID: ndd
Tube:	Panel:
Acquisition Date: 31-May-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	6	0.12	0.12	5.93	5.17	991.59	982.17
UR	5	0.10	0.10	179.89	124.09	132.16	84.14
LL	4976	99.52	99.52	5.43	4.69	3.96	3.49
LR	13	0.26	0.26	494.26	290.23	7.21	4.51

#### IV. RESULTS

To determine which of the mixed lymphocyte hybridoma pools contained significant amounts of NK cell hybridomas,  $1 \times 10^6$  cells/ml hybridoma cells were stained with an anti-DX5 antibody conjugated to a fluorescent label (biotin conjugated rat anti-mouse Pan-NK cell monoclonal antibody and streptavidin-flourescein isothiocyanate (FITC). The fluorescent label (FITC) that was bound to the NK cell hybridomas DX5 surface antigen was detected by a flow cytometer. The results were then analyzed by gating (see Methods). The segment of the dot plot which was to be analyzed was selected for and displayed as a histogram. The mean fluorescence in the selected area was calculated. As shown in Figure 10, the only area analyzed (gated) for mean fluorescence in the first experiment was the lower right portion of the dot plot (R2). The lower right segment is the area that will contain any cell which is labeled with FITC. High amounts of mean fluorescence in the selected area indicated that the anti-DX5-FITC antibody was binding to cells at a higher efficiency. Because DX5 is a cell surface antigen which is unique to NK cells and the secondary streptavidin-FITC conjugate was specific for a biotinylated anti-DX5 antibody, the desirable cell pools displayed high amounts of mean fluorescence in R2. The cell pool which had the highest mean fluorescence and was chosen to be used for further testing (cell pool C6). A histogram was created to show the mean fluorescence in R2 for the Cell pool C6 (Figure 11).

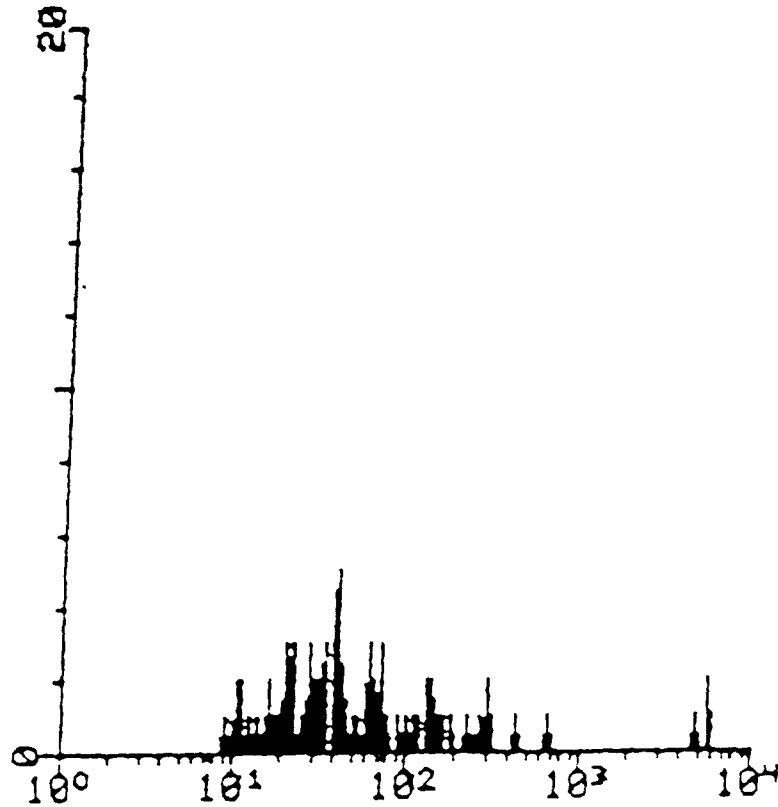
The mixed lymphocyte hybridoma cell pool C6 was then subcloned. Subcloning increases the chance of obtaining a cell pool which contains a single NK cell hybridoma clone. In order to determine which of the single clones prepared from the mixed lymphocyte hybridoma pool C6 were DX5



/S/KTD007

**Figure 11. Histogram of Gate R2 Illustrating the Mean FITC Fluorescence Cell Pool C6:** The logarithmic histogram shows the mean fluorescence intensity emitted by the cells analyzed in gate R2 of Figure 11. As indicated by the arithmetic histogram statistics, 53 total events were accounted for in R2 the mean fluorescence of the cells in R2 was 386.59.

5/KTD007



— Arithmetic Histogram Statistics for 5/KTD007 —

Selected Preferences: Arithmetic/Linear

Parameter FLI-H

Gate G2= R2

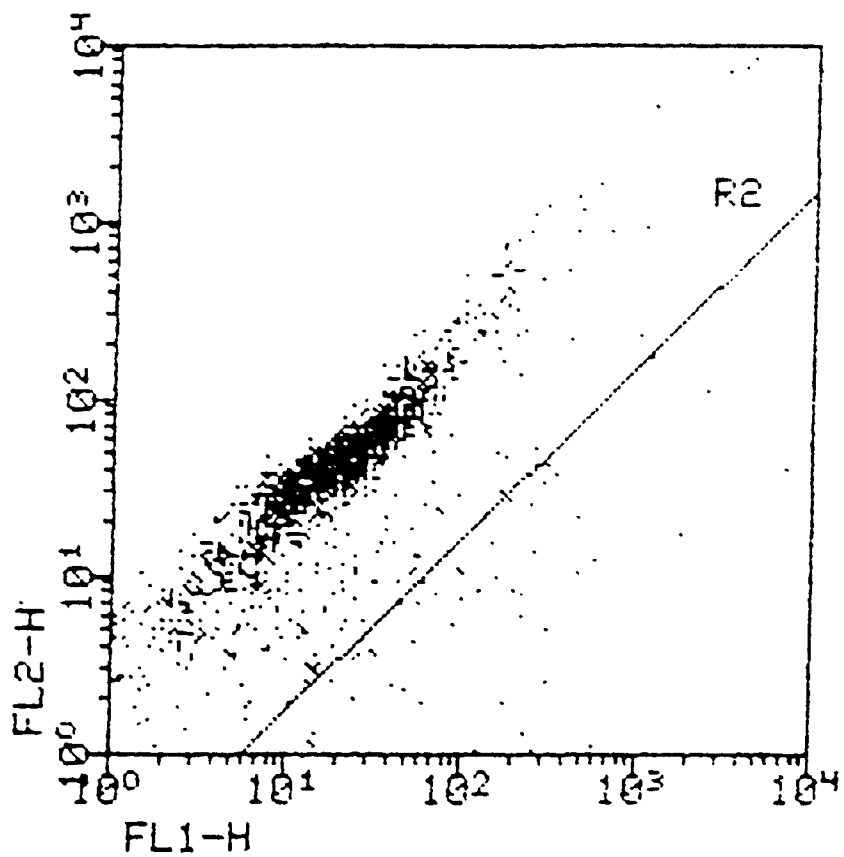
M	Left,Right	Events	%	Peak	PkCh1	Mean	Median	SD	CU %
---	------------	--------	---	------	-------	------	--------	----	------

8	1.00	9646	88	100.00	5	48.68	257.17	48.68	964.61 >100.0
---	------	------	----	--------	---	-------	--------	-------	---------------

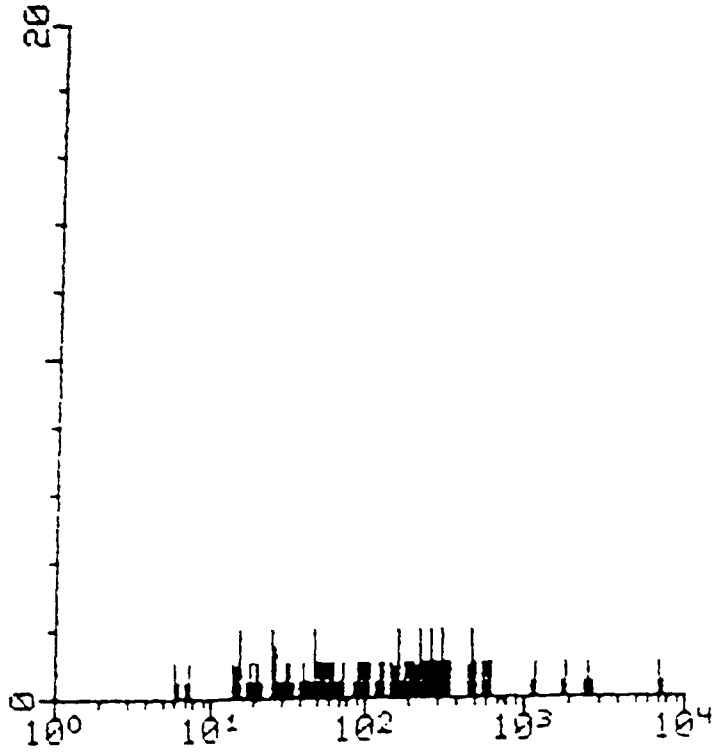
positive, the subclones were tested for the presence of the surface antigen DX5. As can be seen by the dot plot (Figure 12) and histogram (Figure 13) of subclone C6.D2 and dot plot (Figure 14) and histogram (Figure 15) subclone C6.C3, these subclones showed the highest amounts of mean fluorescence in the lower right portion of the plot (gate R2); 386.59 and 522.96, respectively. Because these subclones started out as a single cell in a 96 well plate, we expected to see a high amount of bound FITC label. There were significant amounts of fluorescence detected in gate R2, but not significant enough to satisfy amounts expected if every NK cell were positive. There were also noticeable amounts of cells, indicated by the results shown in Figure 14, detected in the upper right portion of the dot plot. This indicated that the cells were fluorescing at the wavelengths of both FITC and PE.

Because the majority of the subcloned hybridoma cells did not appear to have been labeled with the anti-DX5-FITC conjugate, a test was performed on the hybridoma cell pools C6.D2 and C6.C3 to determine if the clone was contaminated with T cells. The cells were then labeled with an anti-CD3-PE conjugate R-Phycoerythrin (R-PE)-conjugated rat anti-mouse CD3 molecular complex antibody. The dot plot of the analysis of subclone C6.C3 (Figure 16) shows that PE-conjugated antibody is detected in the upper left portions of the dot plot (R2 and R3). If the cells were indeed NK cell hybridomas and not T-cells or NK-T-cells, no fluorescently labeled cells would be detected in the upper left segment. A histogram of each of the gated areas R2 (Figure 17) and R3 (Figure 18) show that unexpected high amounts of mean fluorescence were detected in cell pool C6.C3 (similar results were obtained for cell pool C6.D2). As seen previously, when only FITC labeled antibody was used, fluorescent cells were also detected in the

/4/NDA011



4/NDA011



4/NDA011

— Arithmetic Histogram Statistics for 4/NDA011 ---

Selected Preferences: Arithmetic/Linear

Parameter FL1-H

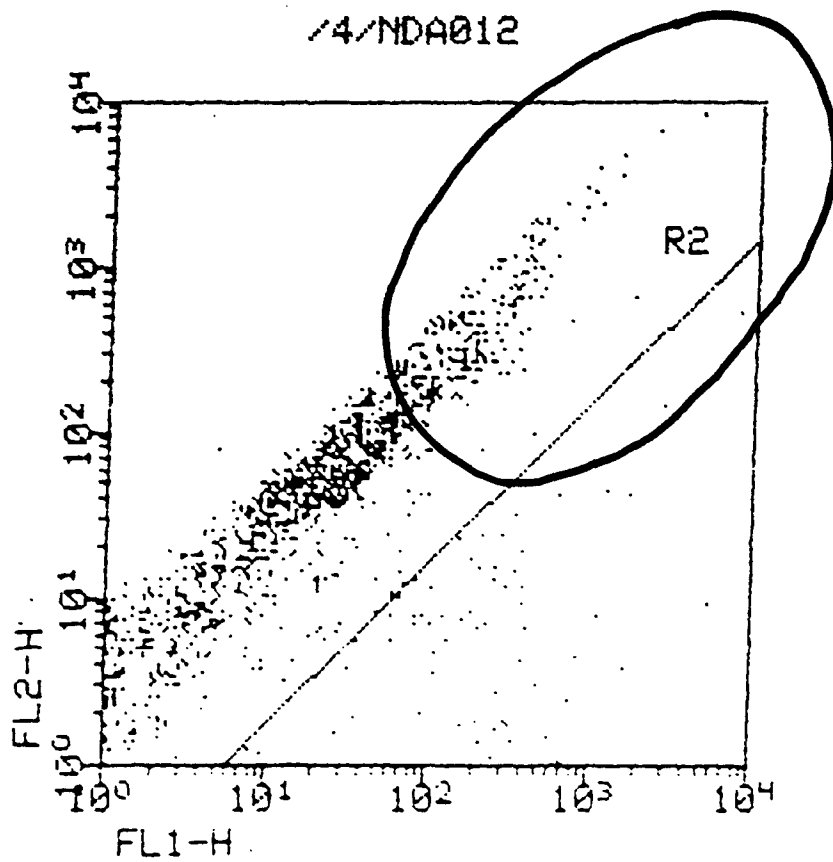
Gate G2= R2

M	Left,Right	Events	%	Peak	PkCh1	Mean	Median	SD	CV %
0	1.00,	9646	53	100.00	2	15.40	386.59	124.09	1018.19 >100.0



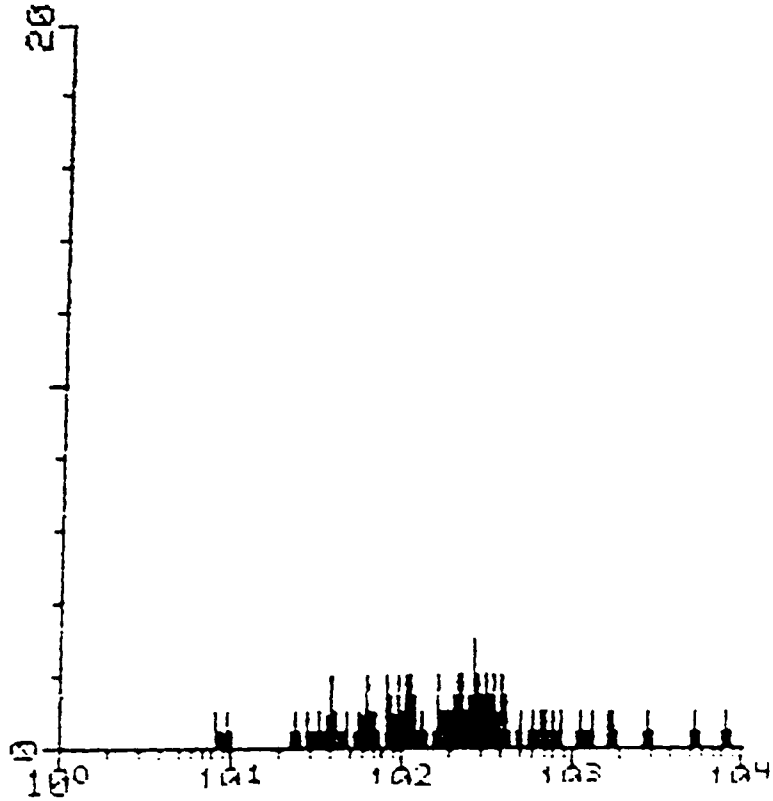
**Figure 14. Gated Dot Plot Analysis of Subclone C6.C3:** Subclone C6.D3 was stained with anti-DX5-FITC and analyzed by flow cytometry. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. The gated area (R2), indicated by the selected triangular area, contains the cells whose mean fluorescence was analyzed with a histogram (see Figure 15). The circled upper right portion of the dot plot indicated cells were fluorescing at wavelengths of both FITC and PE.

/4/NDA012



**Figure 15. Histogram of Gate R2 Illustrating the Mean FITC Fluorescence of Subclone C6.C3:** The logarithmic histogram shows the mean fluorescence intensity emitted by the cells analyzed in gate R2 of Figure 14. As indicated by the arithmetic histogram statistics, 68 total events of 500 were accounted for in R2 the mean fluorescence of the cells in R2 was 522.96.

/4/NDA012



/4/NDA012

--- Arithmetic Histogram Statistics for /4/NDA012 ---

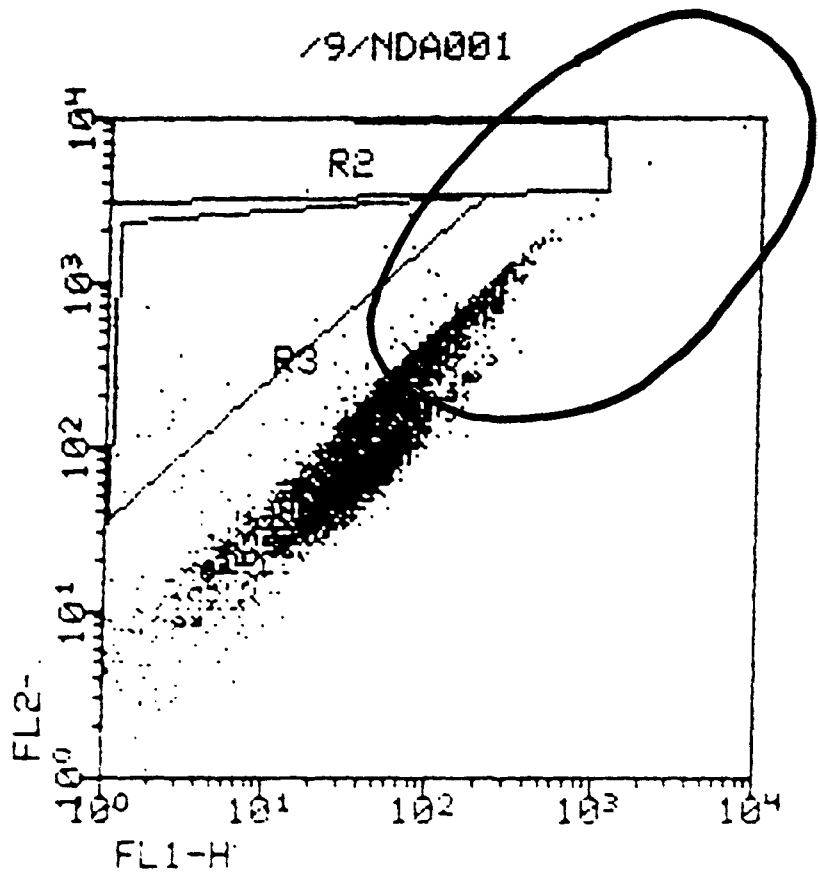
Selected Preferences: Arithmetic/Linear

Parameter FL1-H Gate G2= R2

M	Left,Right	Events	%	Peak	PKCh1	Mean	Median	SD	CU %
0	1.00,	9646	68	100.00	3	264.16	522.96	212.88	1190.85 >100.1

**Figure 16. Gated Dot Plot Analysis of Subclone C6.C3:** Subclone C6.D3 was stained with anti-CD3-PE and analyzed by flow cytometry. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. The gated areas, indicated by the selected rectangular area (R2) (see Figure 17) and the selected triangular area (R3)(see Figure 18), contains the cells whose mean fluorescence was analyzed with a histogram. The circled upper right portion of the dot plot indicated cells were fluorescing at wavelengths of both FITC and PE.

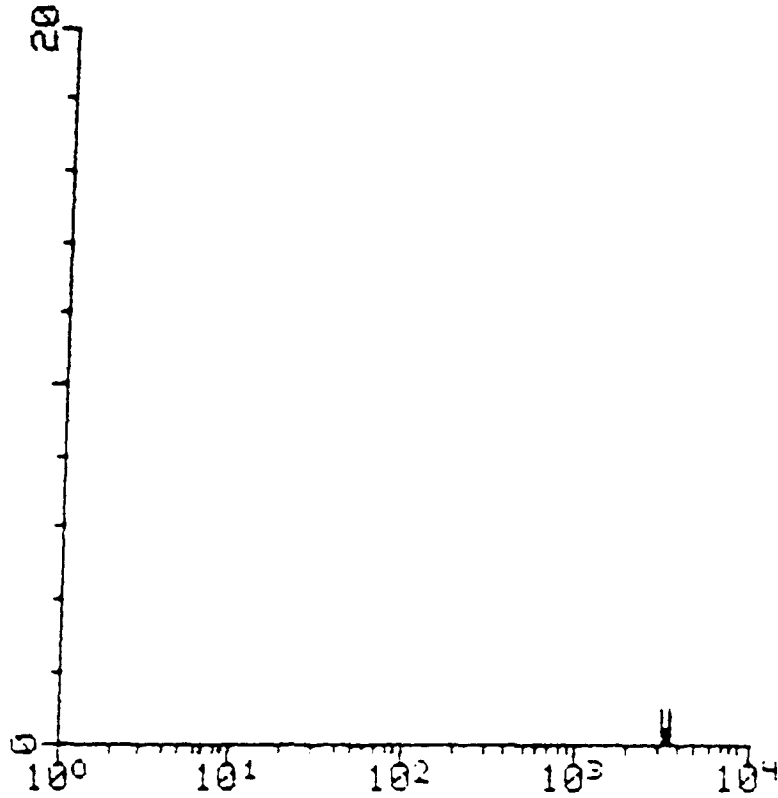
/9/NDA001



**Figure 17. Histogram of Gate R2 Illustrating the Mean PE**

**Fluorescence of Subclone C6.C3:** The logarithmic histogram shows the mean fluorescence intensity emitted by the cells analyzed in gate R2 of Figure 16. As indicated by the arithmetic histogram statistics, 2 total events of 500 were accounted for in R2. The mean fluorescence of the cells in R2 was 3280.24.

/9/NDA001



/9/NDA001

--- Arithmetic Histogram Statistics for /9/NDA001 ---

Selected Preferences: Arithmetic/Linear

Parameter FL2-H Gate G2= R2

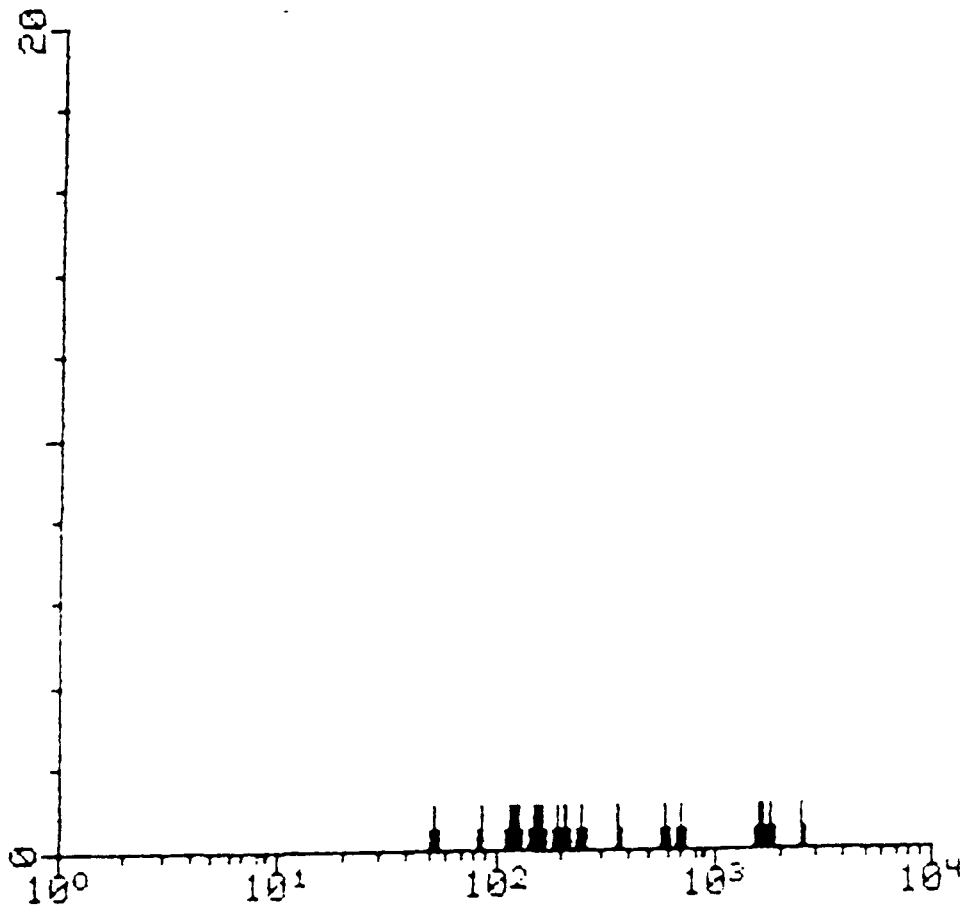
M	Left,Right	Events	%	Peak	PkCh1	Mean	Median	SD	CU %
0	1.00,	9646	2	100.00	1	3162	3280.24	166.83	5.09



**Figure 18. Histogram of Gate R3 Illustrating the Mean PE**

**Fluorescence of Subclone C6.C3:** The logarithmic histogram shows the mean fluorescence intensity emitted by the cells analyzed in gate R3 of Figure 16. As indicated by the arithmetic histogram statistics, 19 total events of 500 were accounted for in R3. The mean fluorescence of the cells in R3 was 557.26.

/9/NDA001



/9/NDA001

--- Arithmetic Histogram Statistics for /9/NDA001 ---

Selected Preferences: Arithmetic/Linear

Parameter FL2-H

Gate G3= R3

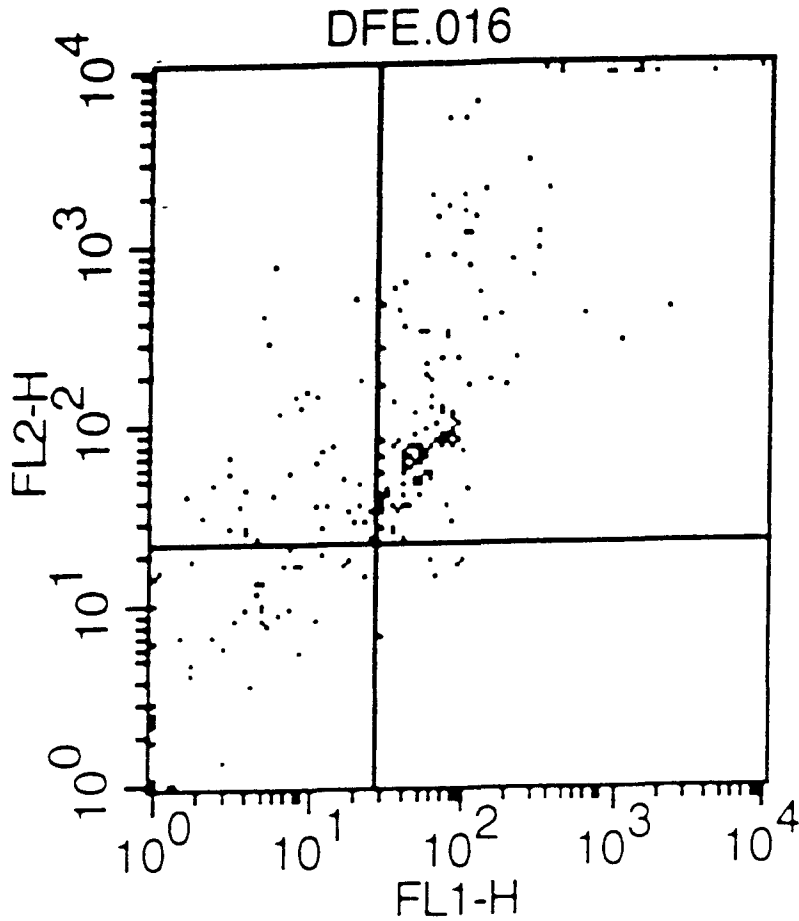
M	Left,Right	Events	%	Peak	PkCh1	Mean	Median	SD	CU %
0	1.00,	9646	100.00	1	52.33	557.26	159.63	713.59	>100.0

upper right portion of the dot plot (Figure 16) indicating that cells were fluorescing at the wavelengths expected for both FITC and PE. These results indicated one or a combination of three things: 1. the cells were contaminated with T cells, 2. the cells were actually NK-T cells, or 3. The cells were autofluorescent. These possibilities were explored in further experiments.

To determine whether or not the hybridoma cells which were being screened were positive for both CD3 and DX5, which would suggest an NK-T cell was actually created, another experiment was conducted in which the cells were labeled with both the PE-conjugate and the FITC conjugate simultaneously. This time all four segments of the dot plot were gated in order to determine the mean fluorescence detected in each area. After reviewing the mean fluorescence of the lower right quadrant (Q-LR) of the dot plot (Figure 19), it was determined that hybridoma cell pool C6.C3 had minimal amounts of FITC labeled cells. Also illustrated by Figure 20, the upper left quadrant (Q-UL) displayed higher amounts of PE labeled cells, and significantly higher amounts of cells which were fluorescing at the wavelength of both FITC and PE as indicated by the mean fluorescence of the upper right quadrant (Q-UR). As a control in this experiment, hybridoma cells and myeloma cells, which were unlabeled, were also passed through the flow cytometer. The unlabeled hybridoma subclones (Figure 20) and the unlabeled P3X (Figure 21) also showed significant amounts of cells in Q-UR. This indicated that the cells had autofluorescent properties causing them to appear to be labeled with FITC and PE.

Because each of the tests showed significant amounts of fluorescence in Q-UR, and this was highly indicative of autofluorescence, the problem of autofluorescence was addressed. Two different methods for eliminating cellular autofluorescence were tested. In the first, a modification of the

**Figure 19. Gated Dot Plot Analysis of Subclone C6.C3:** Subclone C6.C3 was stained with both anti-DX5-FITC and anti-CD3-PE and analyzed by flow cytometry. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



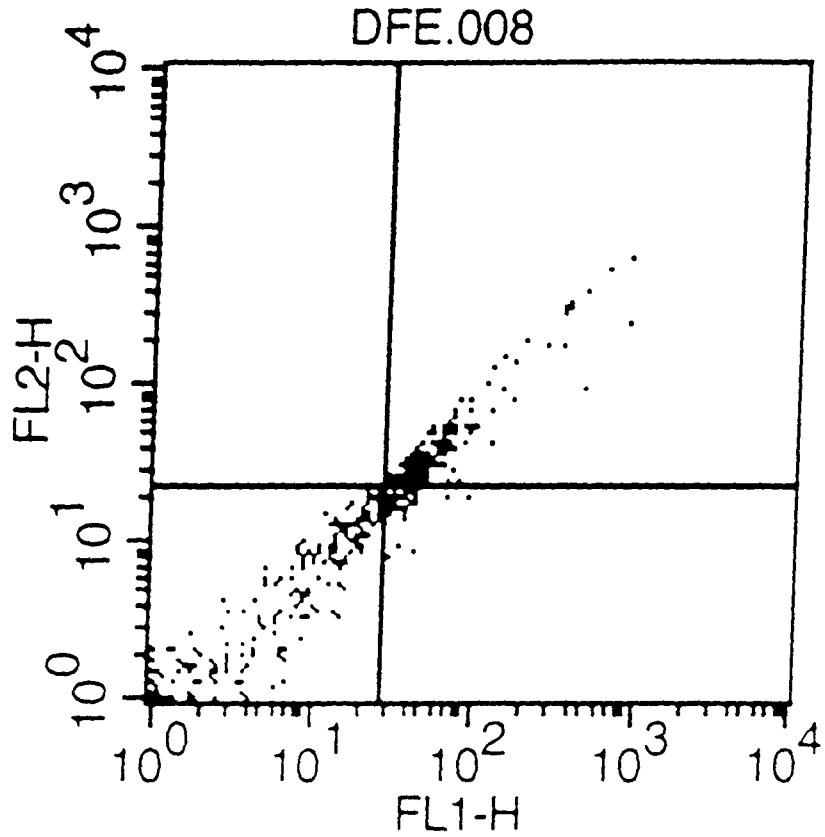
Quadrant Statistics

File: DFE.016	Log Data Units: Linear Values
Sample ID: DFE	Patient ID: DFE
Tube:	Panel:
Acquisition Date: 30-Mar-00	Gate: No Gate
Gated Events: 240	Total Events: 240
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	41	17.08	17.08	11.49	8.79	103.13	61.61
UR	142	59.17	59.17	256.62	84.77	1255.54	212.07
LL	52	21.67	21.67	4.32	2.64	7.74	4.95
LR	5	2.08	2.08	69.18	64.01	14.39	13.63

**Figure 20. Gated Dot Plot Analysis of Unlabeled Hybridoma Cells:**

Unlabeled hybridoma cells (subclone C6.C3) were analyzed by flow cytometry. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



Quadrant Statistics

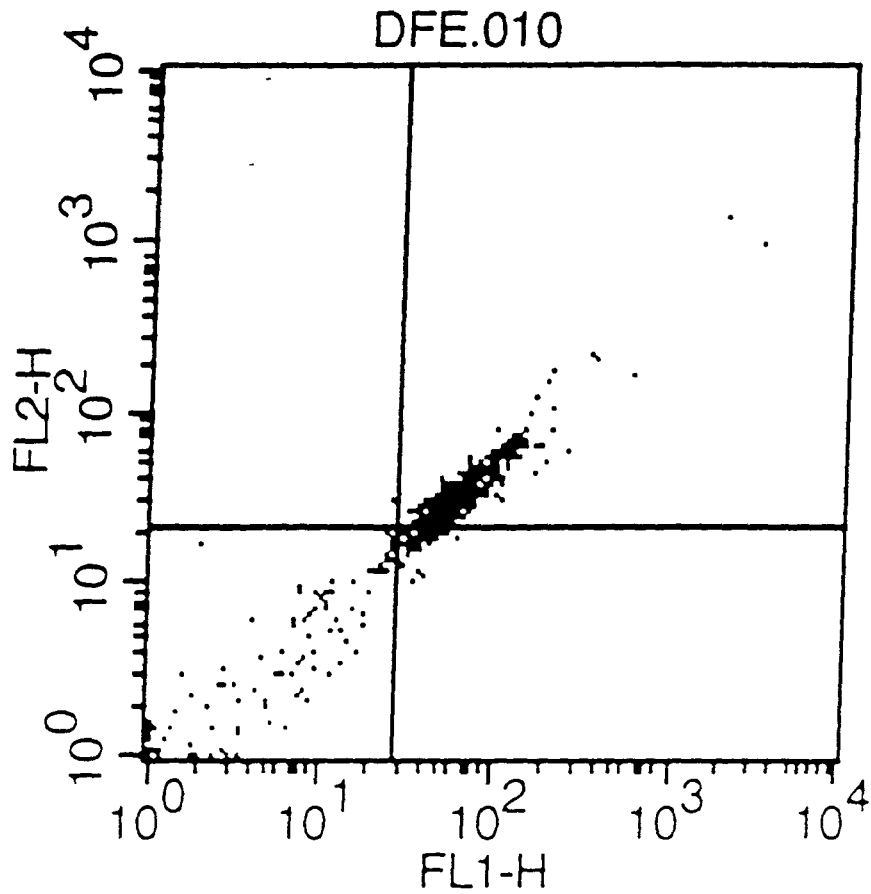
File: DFE.008  
 Sample ID: DFE  
 Tube:  
 Acquisition Date: 30-Mar-00  
 Gated Events: 440  
 X Parameter: FL1-H (Log)  
 Quad Location: 26, 22

Log Data Units: Linear Values  
 Patient ID: DFE  
 Panel:  
 Gate: No Gate  
 Total Events: 440  
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	0	0.00	0.00	...	...	...	...
UR	159	36.14	36.14	87.76	61.66	58.09	41.31
LL	223	50.68	50.68	9.47	5.86	6.34	4.03
LR	58	13.18	13.18	37.56	36.36	18.12	17.72

**Figure 21. Gated Dot Plot Analysis of Unlabeled P3X cells:** Unlabeled P3X cells were analyzed by flow cytometry. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.





Quadrant Statistics

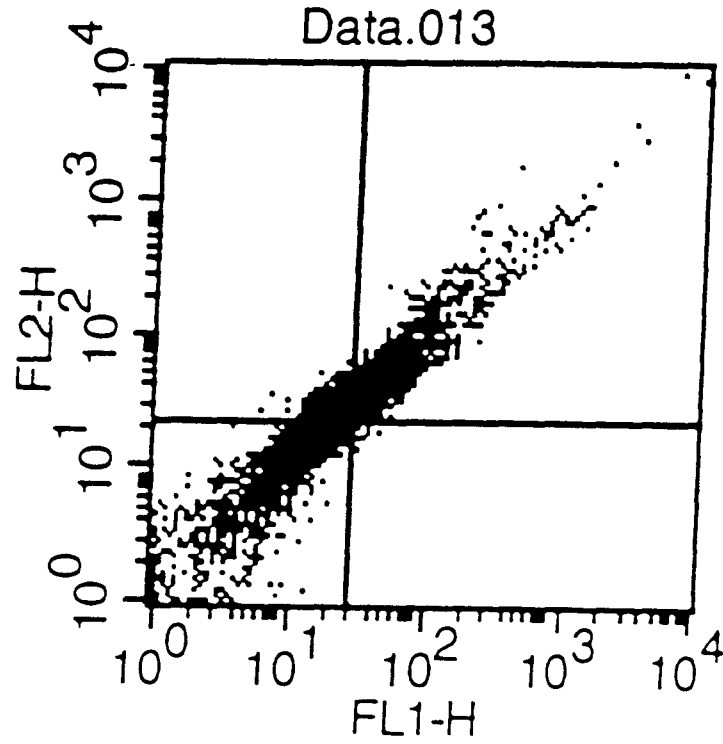
File: DFE.010	Log Data Units: Linear Values
Sample ID: DFE	Patient ID: DFE
Tube:	Panel:
Acquisition Date: 30-Mar-00	Gate: No Gate
Gated Events: 540	Total Events: 540
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1	0.19	0.19	24.58	24.58	30.51	30.51
UR	353	65.37	65.37	84.82	65.88	46.90	38.19
LL	117	21.67	21.67	8.69	5.06	5.47	3.46
LR	69	12.78	12.78	36.36	35.88	18.99	18.76

experiment conducted by Halden *et al.*, hybridoma cells were fixed with paraformaldehyde and permeabilized with the detergent n-octyl-B-D-glucopyranoside (OG) and then treated with various concentrations (0.01mg/ml-2mg/ml) of the quenching dye trypan blue. As shown by comparing Figure 22 representing cells treated with 0.01µg/ml trypan blue with Figure 23 representing cells treated with 2µg/ml trypan blue, there was no significant difference between the mean fluorescence levels any of the cell populations treated with any concentration of trypan blue. There was also no significant difference between the C6.C3 cells treated with 10mg/ml trypan blue (Figure 24) and the C6.C3 cells which were untreated (Figure 25).

A second experiment was performed in order to compensate for the autofluorescence emitted by the hybridoma cells. A modification of the method employed by Moisman and colleagues was used for this experiment. Cytofix-Cytoperm™ was used to fix and permeabilize the cells and a special washing agent Perm Wash was used to wash the cells while keeping the membranes permeable to the trypan blue. After fixation and permeabilization, hybridoma subclone C6.C3 was treated with increasing concentrations (.01mg/ml-10mg/ml) of the quenching dye trypan blue. As shown by comparing Figure 26 representing cells treated with 0.01µg/ml trypan blue with Figure 27 representing cells treated with 10µg/ml trypan blue, there was no significant difference between the mean fluorescence levels any of the cell populations treated with any concentration of trypan blue. There was also no significant difference between the C6.C3 cells treated with trypan blue (Figure 26) and the C6.C3 cells which were untreated (Figure 28).

**Figure 22. Gated Dot Plot Analysis of Subclone C6.C3 Treated with 0.01 $\mu$ g/ml of Trypan Blue:** Subclone C6.C3 was fixed and permeabilized with paraformaldehyde and 0.06% OG, respectively. The cells were then treated with 0.01 $\mu$ g/ml of trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.

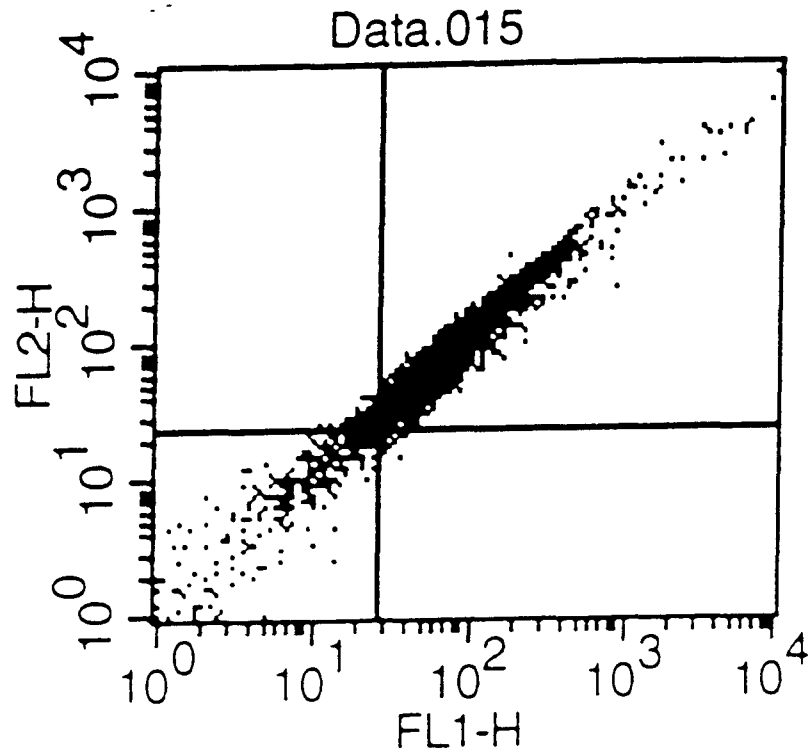


Quadrant Statistics

File: Data.013	Log Data Units: Linear Values
Sample ID: ndd	Patient ID: ndd
Tube:	Panel:
Acquisition Date: 31-May-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1144	22.88	22.88	20.96	20.56	29.76	29.20
UR	2019	40.38	40.38	79.02	48.24	85.88	53.44
LL	1805	36.10	36.10	12.17	10.18	13.52	11.33
LR	32	0.64	0.64	31.25	30.95	20.16	20.03

**Figure 23. Gated Dot Plot Analysis of Subclone C6.C3 Treated with 2 $\mu$ g/ml of Trypan Blue:** Subclone C6.C3 was fixed and permeabilized with paraformaldehyde and 0.06% OG, respectively. The cells were then treated with 2 $\mu$ g/ml of trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.

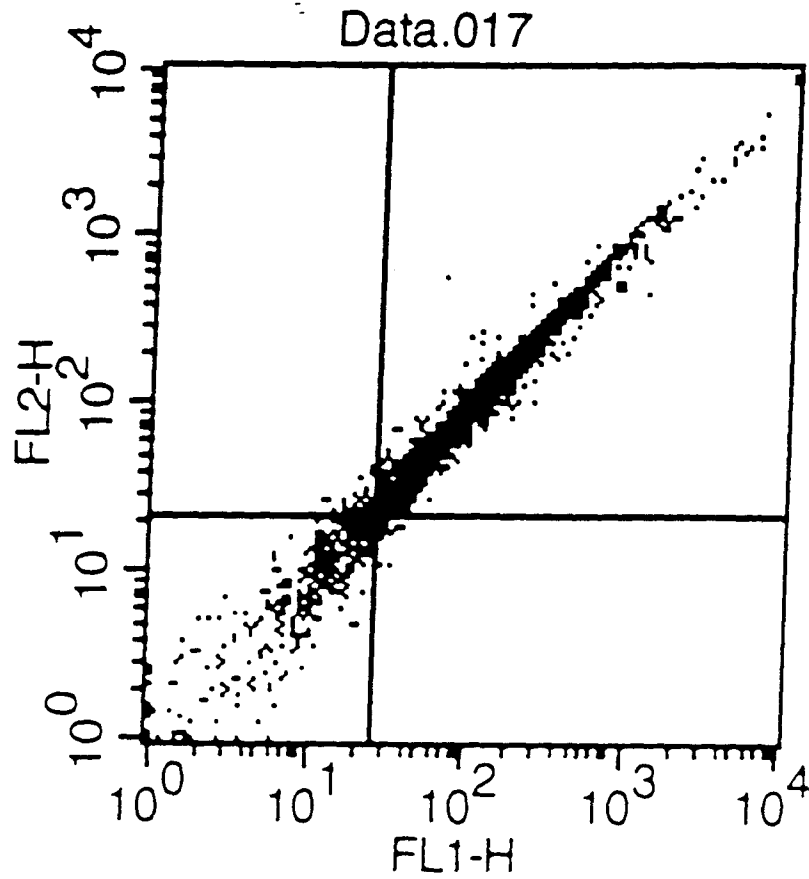


Quadrant Statistics

File: Data.015	Log Data Units: Linear Values
Sample ID: ndd	Patient ID: ndd
Tube:	Panel:
Acquisition Date: 31-May-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	147	2.94	2.94	22.34	22.04	27.85	27.55
UR	4505	90.10	90.10	115.98	82.59	129.14	94.26
LL	325	6.50	6.50	11.48	8.77	11.42	8.94
LR	23	0.46	0.46	30.68	30.46	19.40	19.26

**Figure 24. Gated Dot Plot Analysis of Subclone C6.C3 Treated with 1µg/ml of Trypan Blue:** Subclone C6.C3 was fixed and permeabilized with paraformaldehyde and 0.06% OG, respectively. The cells were then treated with 1µg/ml of trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



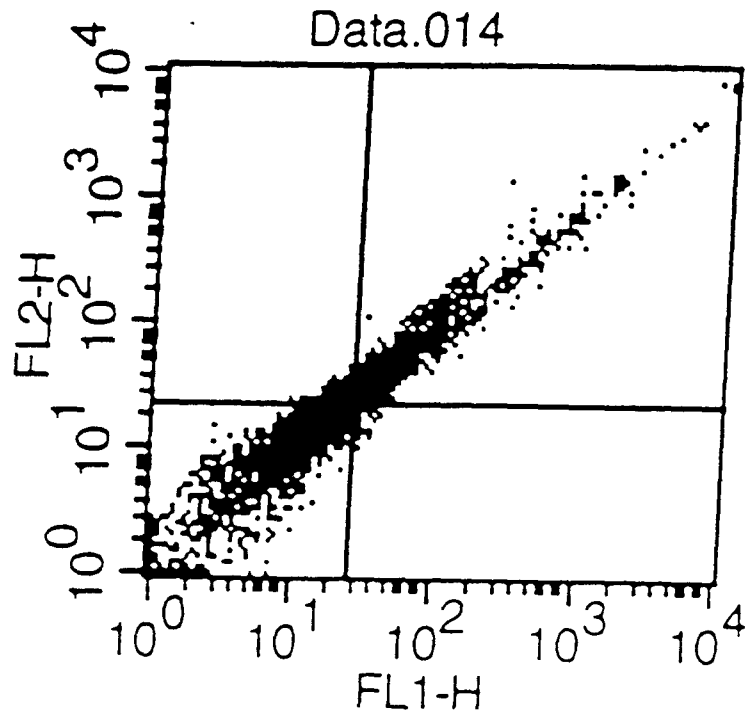
Quadrant Statistics

File: Data.017	Log Data Units: Linear Values
Sample ID: ndd	Patient ID: ndd
Tube:	Panel:
Acquisition Date: 31-May-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	57	1.14	1.14	22.00	21.65	26.90	26.53
UR	4516	90.32	90.32	175.20	122.01	163.84	115.23
LL	386	7.72	7.72	12.69	9.72	10.49	7.91
LR	41	0.82	0.82	31.72	31.51	19.31	19.08



**Figure 25. Gated Dot Plot Analysis of Subclone C6.C3:** As a control, subclone C6.C3 was left untreated with paraformaldehyde, OG and with trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.

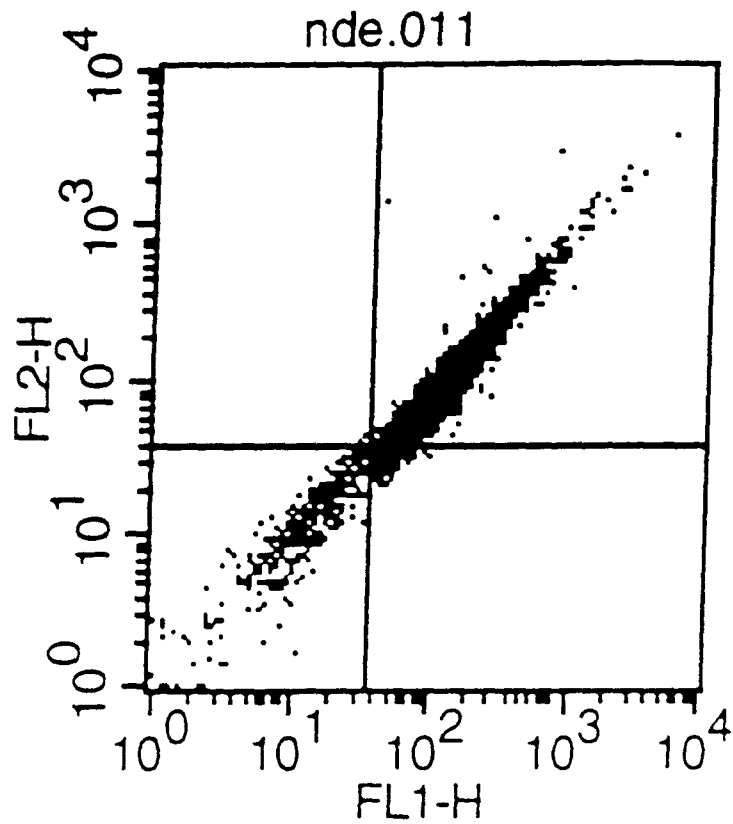


Quadrant Statistics

File: Data.014	Log Data Units: Linear Values
Sample ID: ndd	Patient ID: ndd
Tube:	Panel:
Acquisition Date: 31-May-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 26, 22	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	633	12.66	12.66	22.09	21.80	26.20	26.02
UR	1745	34.90	34.90	122.10	54.75	106.35	52.03
LL	2541	50.82	50.82	13.18	10.95	13.40	11.25

**Figure 26. Gated Dot Plot Analysis of Subclone C6.C3 Treated with 0.01 $\mu$ g/ml of Trypan Blue:** Subclone C6.D3 was fixed and permeabilized with Cytoperm/Ctyofix™. The cells were then treated with 0.01 $\mu$ g/ml of trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



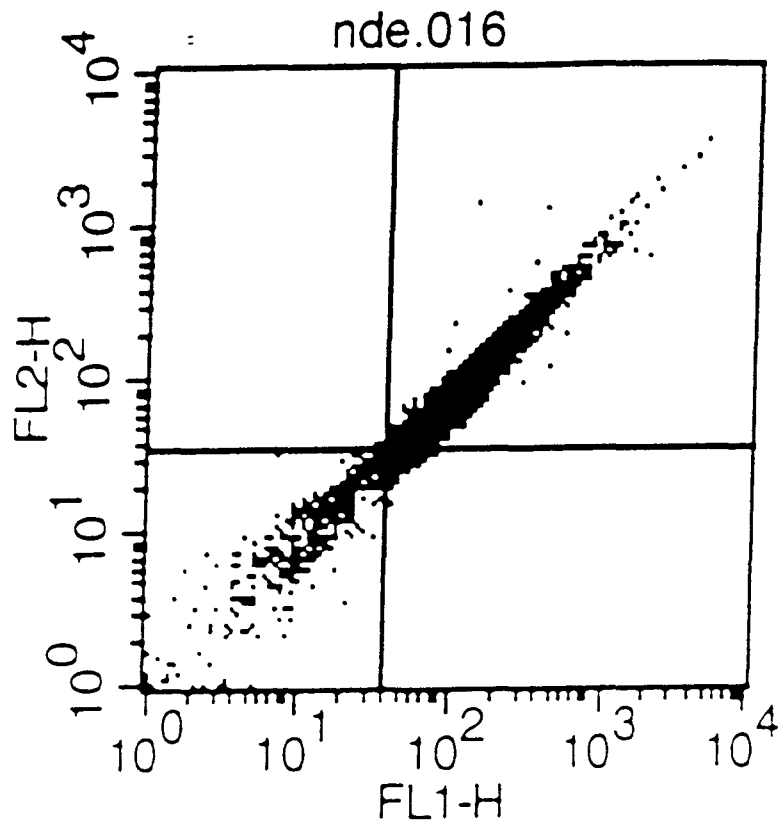
Quadrant Statistics

File: nde.011  
 Sample ID: nde  
 Tube:  
 Acquisition Date: 06-Jul-00  
 Gated Events: 5000  
 X Parameter: FL1-H (Log)  
 Quad Location: 37, 34

Log Data Units: Linear Values  
 Patient ID: nde  
 Panel:  
 Gate: No Gate  
 Total Events: 5000  
 Y Parameter: FL2-H (Log)

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	6	0.12	0.12	32.45	32.00	38.64	38.54
UR	4404	88.08	88.08	152.24	124.50	115.74	89.90
LL	379	7.58	7.58	16.27	12.89	13.78	10.79
LR	211	4.22	4.22	52.48	51.76	30.23	30.02

**Figure 27. Gated Dot Plot Analysis of Subclone C6.C3 Treated with 10 $\mu$ g/ml of Trypan Blue:** Subclone C6.D3 was fixed and permeabilized with Cytoperm/Ctyofix™. The cells were then treated with 10 $\mu$ g/ml of trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.

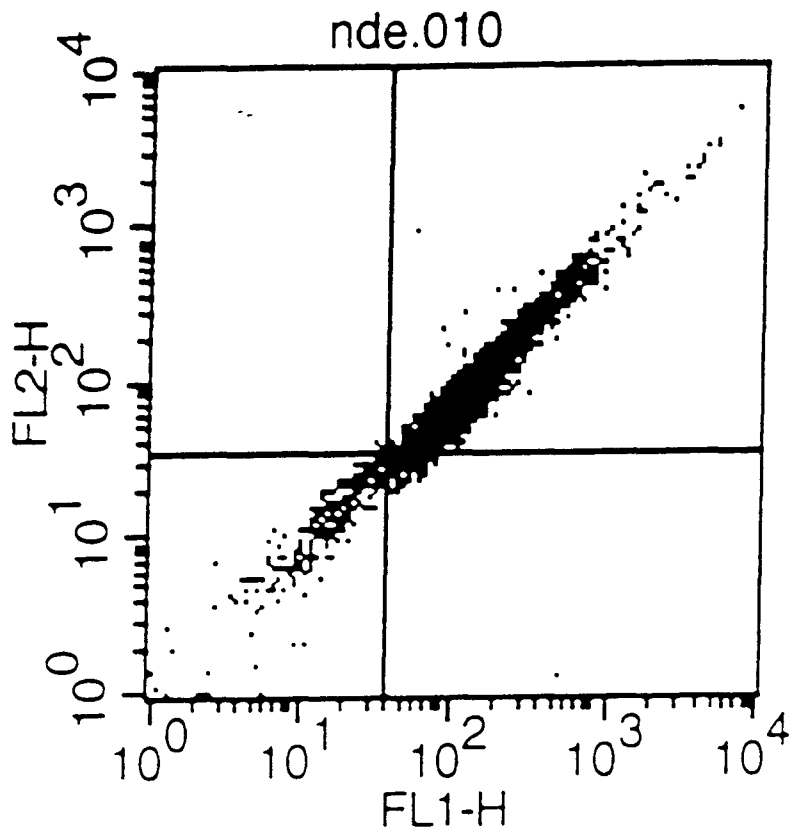


Quadrant Statistics

File: nde.016	Log Data Units: Linear Values
Sample ID: nde	Patient ID: nde
Tube:	Panel:
Acquisition Date: 06-Jul-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 37, 34	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	9	0.18	0.18	32.37	32.26	39.37	38.47
UR	4286	85.72	85.72	140.51	113.36	108.50	84.48
LL	451	9.02	9.02	16.06	12.39	13.64	10.48
LR	254	5.08	5.08	49.74	49.13	30.30	30.08

**Figure 28. Gated Dot Plot Analysis of Subclone C6.C3:** As a control, subclone C6.C3 was left untreated with Cytoperm/Ctyofix™ and trypan blue. The x-axis, FL1-H, represents the fluorescence intensity emitted by cells labeled with FITC. The y-axis, FL2-H, represents the fluorescence intensity emitted by cells labeled with PE. The graph is on a logarithmic scale. There are four gated regions: Q-UL, Q-UR, Q-LL and Q-LR (see Figure 4). The numerical mean fluorescence intensity of each gate is analyzed in the quadrant statistics located below the dot plot.



Quadrant Statistics

File: nde.010	Log Data Units: Linear Values
Sample ID: nde	Patient ID: nde
Tube:	Panel:
Acquisition Date: 06-Jul-00	Gate: No Gate
Gated Events: 5000	Total Events: 5000
X Parameter: FL1-H (Log)	Y Parameter: FL2-H (Log)
Quad Location: 37, 34	

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	8	0.16	0.16	29.81	27.63	47.69	42.74
UR	4524	90.48	90.48	170.10	132.76	127.14	94.61
LL	262	5.24	5.24	18.05	14.71	15.30	12.24
LR	206	4.12	4.12	54.76	52.55	30.31	29.77



## V. DISCUSSION

Many laboratories have studied NK cell function by using purified NK cell preparations (Wigzell and Ramstedt, 1989; Fagan *et al.* 1991). In these studies, human peripheral blood lymphocytes were aseptically collected by venous puncture. A standard Ficoll-Isopaque gradient was used to separate the components of the blood into their constitutive parts. The mononuclear cells were removed from suspension and were treated with 4.5ml of distilled water to lyse the residual red blood cells (PBS was later added to solution to restore isotonicity). The remaining adherent cells (macrophages) were then removed by incubating cells in plastic dishes for 60 minutes at 37°C. The non-adherent cells were then collected and added to a nylon-wool lined 20ml syringe, pre-incubated with media. The cells were eluted from the syringe by successive application of 2mls of warm medium. The cells that remained after the application to nylon wool were then separated by Percoll density gradient centrifugation. To further purify the cells, T cells were removed from the cell population by rosetting (incubation with sheep red blood cells followed by removal of bound cells by Ficoll density gradient centrifugation). After this laborious process was complete, 10% of the cells which remained were determined to be T-cells. Also, approximately 50% of the NK cells were lost during the nylon wool purification step.

As described, the separation of NK cells from other lymphocytes, T cells in particular, in mixed lymphocyte culture is a very difficult one to perform. Also, because lymphocytes, NK cells included, do not survive for long periods of time in culture, when NK cells are isolated from mixed lymphocyte cultures they are not in very high yield. So, it is highly possible that many, if not all of the laboratories that have employed similar or exact

techniques as Fagan *et al.* in their purification of NK cells, have actually had T cells or NK-T cells contaminating their cultures. NK cell clones have been believed to have been created in multiple laboratories, but the task of creating NK cell clones is not only a tedious one, but a very expensive one as well. Also, NK cell clones raised in culture have displayed many characteristics of T cells. So, the probability that the clones created were actually T cells with NK like characteristics or, that the cells were actually NK-T cells could not be ruled out.

The work in this laboratory was designed to successfully create, identify and clone a natural killer cell hybridoma. The creation of a pure NK cell hybridoma clone, rather than a NK cell clone, may be the best means of obtaining a testable pool of NK cells. Other laboratories have established hybridoma cells with NK like properties and function (Suzuki *et al.* 1992, and Kubota *et al.* 1996). Yasumoto Suzuki and his colleagues produced NK hybridomas and tested them for NK like activity against syngenic tumor cells (Suzuki *et al.* 1992). This team of researchers obtained lymphocytes from BALB/c nude mice. The advantage of using nude mice as opposed to other strains, such as the Swiss Webster strain used in our studies, is that nude mice lack T-cells derived in the thymus. The lack of T-cells in nude mice is due to an atrophy of the thymus. Although the majority of the T-cell population develop in the thymus, there are other T-cells, mainly found in the peripheral blood and the intestinal mucosa, that do not require maturation in the thymus. Included in this category are  $\gamma\delta$ T-cells and NK-T cells. So, although the use of BALB/c nude mice greatly reduced the chances of T-cell hybridoma contamination of NK cell hybridomas, the use of this mouse strain could not absolutely prevent T-cell hybridomas from being created.

Suzuki et al fused splenocytes of BALB/c nude mice with the mouse myeloma cell (cancerous plasma cell) line NS-1 and cultured the cells in HAT medium. The remaining hybridoma cells were screened for the presence of the Asialo-GM1 (ASGM1) receptor using an indirect immunofluorescence method. The hybridoma cells created by Suzuki and colleagues were also screened for the presence of T-cell markers L3T4 and Lyt-2 by flow cytometry. The cells were negative for such T-cell markers. However, surface antigens responsible for the recognition of the cytokine IL-2, p55 and p75, were not present on the surface of the cell. IL-2 is a cytokine which is responsible for increased NK cell responses. Functional NK cells have been identified as L3T4<sup>-</sup>, Lyt-2<sup>-</sup>, ASGM1<sup>+</sup>, p55 IL-2 receptor<sup>-</sup> and p75 IL-2 receptor<sup>+</sup>. In these studies conducted by Suzuki et al, the NK-like hybridomas were found to have the ability to lyse both YAC-1 target cells and RL $\sigma$ -1 cells with the same intensity of cloned NK cells. Although the NK-like hybridoma cell lines created by Suzuki and colleagues had several phenotypic properties of NK cells, such as the absence of L3T4 and Lyt-2 and the presence of ASGM-1, the possibility the created clones were actually unresponsive lymphokine activated killer cells (cells with NK like function that can only kill subsequent to activation by secreted cytokines) could not be ruled out.

The hybridoma that was created by Suzuki *et al.* could have been an NK cell, but the NK hybridoma that was created was not normal in its responses to IL-2. The clone that was created lacked an IL-2 receptor, so its phenotypic characteristics were more like that of a resting NK cell than of an active one. There were also no subsequent publications from Suzuki suggesting no further studies were completed using their NK hybridoma clone. This indicates that the cell lines could not be maintained in the lab.

Another team of researchers led by Koichi Kubota (Kubota *et al.*, 1996 and 1997) also created a 'novel' killer hybridoma that had some NK-like characteristics. This hybridoma was created in order to study the mechanism of MHC class I unrestricted killing. The T-cell lymphoma YACUT was fused with T-cells that were CD8 $\alpha^+\beta^+$  (the cells had both molecules that make up the CD8 heterodimer) and Fc $\gamma$ RIII (CD16) negative. The properly fused cells were selected for by culturing in HAT media and the analysis of cell surface antigens was conducted by flow cytometry analysis. The fusion of these two cell lines produced some hybrids that were not T-cells and were not MHC restricted. The cells that were produced, however, displayed properties of both T-cells and NK cells, which is characteristic of NK-T cells. All fused hybridoma cells had a Fc $\gamma$ II/III (CD16) receptor, which is characteristic of NK cells (as well as some phagocytic cells) and responsible for the NK cell's ADCC property. All strains were CD4 $^-$  and CD8 $\alpha^+\beta^-$  and TCR $\alpha\beta^+$  indicating that the cells also had some T-cell characteristics. Since this fusion produced unexpected hybrids that were not only TCR $\alpha\beta^+$  but also Fc $\gamma$ RIII $^+$ , this killer hybridoma was not an NK cell hybridoma, but was an NK-T cell hybridoma.

Both of the previously described studies were successful in the creation of a hybridoma with NK-like activity. However, neither was able to isolate pure NK cell hybridoma pools. As there are no currently available NK clones, our lab also attempted to produce NK hybridomas. In order to positively identify NK hybridoma cells, testing for the presence of surface antigens can be conducted. Antibodies have been used in previous studies to identify NK cells based upon the surface antigens present, such as, ASGM1 and NK1.1, but these antibodies did not seem to be as useful for

our studies. An anti-ASGM1 antibody was used by Suzuki and colleagues to identify NK hybridoma clones. Previous work in our laboratory by Sharon L. Usip (unpublished data), has shown that antibodies to ASGM1 bind with very high efficiency, but also bind very non-specifically. Kasai and associates reported that NK cells express high amounts of ASGM1 on their surfaces and will therefore bind to anti-ASGM1 with high efficiency (Kasai *et al.*, 1980). However, they also found that some peripheral blood T-cells express a glycolipid, GM1, on their surface which will also bind anti-ASGM1. It has also been reported that approximately 10-14% of adult murine thymocytes express the ASGM1 antigen on their surface (Tuttles *et al.*, 1987). Further testing with anti-ASGM1 antibodies have shown that anti-ASGM1 will also bind with low affinity to macrophages (Wiltrout *et al.*, 1985). Although GM1 is present only in low densities on the surface of T-cells and binds with low affinity to macrophages, the probability that use of an anti-ASGM1 antibody will yield the positive staining of T-cells and macrophages must be taken into consideration.

Another antibody, which has been used to identify NK cells in mixed lymphocyte culture, is anti-NK1.1 (Koo *et al.*, 1984). NK1.1 is a surface antigen found on most mouse NK cell surfaces, however, NK1.1 was not found on cells originally used in these studies. In addition, the surface antigen NK1.1 has also been identified on a small sub-population of T-cells and monocytes (Yu *et al.*, 1992 and Vicari *et al.*, 1996).

Linked to the NK1.1 surface marker and present on the surface of NK cells belonging to mice which do not express NK1.1, is the DX5 surface antigen (Moretta *et al.*, 1997). According to the technical data sheet provided by BD Pharmingen, the antibody against NK cell surface antigen DX5, biotin conjugated rat anti-mouse Pan-NK cell (anti-DX5), is a

relatively new antibody but has been effectively used to identify NK cells in freshly isolated mixed lymphocyte cultures. There have been no studies to date that used the biotin conjugated rat anti-mouse Pan-NK cell (anti-DX5) in hybridoma studies. For the previously mentioned reasons, the antibody which seemed to be the best choice for these studies, even though it is a low affinity binding antibody, was DX5.

The company reported and unpublished results (provided by Kathy Thompson of our laboratory) confirmed that the anti-DX5 antibody could not be used in immunohistochemistry studies due to low affinity or sensitivity of the antigen to fixation methods. Previous studies using fluorescently labeled anti-DX5 antibody, conducted by BD Pharmingen, have shown successful labeling of NK cells in spleen cell preparations and analysis of NK cells using Flow cytometry.

To develop a NK cell clone, a standard fusion was performed between freshly isolated spleen cells and P3X non-secreting mouse myeloma cells (cancerous plasma cells). After properly fused cells were selected for in HAT media, the hybridoma cell pools were tested for the presence of surface antigen DX5. Antibody staining and subsequent flow cytometry analysis identified mixed lymphocyte hybridoma cell pools which yielded high amounts of mean fluorescence with FITC-labeled anti-DX5. The cell pool with the highest mean fluorescence was chosen for further investigation. Mixed lymphocyte hybridoma cell pool C6 was chosen to be subcloned by serial dilution in a 96 well plate. The subclones in the wells of the 96 well plate which contained one cell per well were then retested for the presence of the NK cell surface antigen DX5. It was expected that the subcloned cells which stained positive for DX5 would have an extremely high mean fluorescence. This is because the cells tested were thought to be single

clones, and since all NK cells are thought to have DX5 on their surface, and the clone should have been a clone of one cell, the entire population, theoretically should have been staining positive with high frequency and have a high mean fluorescence. This, however, was not the case. The results of the dot plot and histogram analysis of the fluorescence of these subclones (see Figures 12-15) showed cell staining at a mediocre level and a relatively moderate mean fluorescence.

To determine if the clones were contaminated with T cells, the subclones were stained with both FITC-anti-DX5 and PE-anti-CD3. The dot plot analysis of the fluorescence of these subclones showed that the cells were fluorescing at the wavelengths of both FITC and PE (see Figure 16). This could have occurred for multiple reasons. If T cells were contaminating the wells of the subclones, the PE-labeled anti-CD3 would be binding to the CD3 on the surface of the T-cells and therefore detected by the flow cytometer. There is also the possibility that the subclones were NK-T cells and had both CD3 and DX5 on their surface. Finally, the detection of fluorescence at both wavelengths could have been due to autofluorescence.

A common problem in the detection of fluorescent markers lies in the fact that cells have inherent fluorescent properties (Viksman *et al.*, 1994). When a cell emits light naturally by an unstained particle, that cell is said to be autofluorescent. Autofluorescence of cells, sometimes referred to as background noise, makes it hard to distinguish which detected signals are coming from fluorescent labels and which are coming from components within the cell, such as flavins and pyridine nucleotides, that cause autofluorescence (Wede, *et al.*, 1997). The amount of autofluorescence that a cell emits depends upon the type of cellular component involved, the

wavelength of the laser beam, and the wavelength of reactivity of the marker being analyzed (Nguyen *et al.*, 2000).

In these studies, the cells were examined by fluorescence microscopy in order to determine if the PE-anti-CD3 and FITC-anti-DX5 were binding to the same cell. Autofluorescence was present at all wavelengths with these cells. Cells were also viewed, which had not been stained, under the microscope. These cells were also fluorescing at all wavelengths. This confirmed that the problems encountered were due to the autofluorescence of the hybridoma cells. Methods for eliminating autofluorescence were then investigated.

Because autofluorescence stems from components found within the cell, the cell membrane must be made permeable to autofluorescence quenching dyes. It has been a common practice to fix and permeabilize cells in order to allow for the successful staining of intracellular antigens (Moisman *et al.*, 1997). Similar techniques are also used to quench the autofluorescence of intracellular components (Halden *et al.*, 1991, Vicksman *et al.*, 1994 and Moisman *et al.*, 1997). There have been no studies to date that have addressed the problem of autofluorescence in hybridoma cells or of NK cells. However, there have been several studies dealing with correcting the autofluorescence of macrophages, another key player in the immune defenses.

In an attempt to compensate for the problem of autofluorescence, the final experiments were modeled after two protocols used by Moisman *et al.* and Halden *et al.* (Halden *et al.*, 1991 and Moisman *et al.*, 1997). The protocols used by the previously mentioned laboratories corrected the problem of autofluorescence in human macrophages. In the first case, Halden and colleagues investigated methods for quenching cellular



autofluorescence in human macrophages. Human macrophages from the peripheral blood and were stained with fluorescently labeled antibodies. After cell staining, the cells were subjected to three different methods of crystal violet treatment. One pool of cells (pool A) was treated with crystal violet only. Pool B was permeabilized with the detergent, n-octyl-B-D-glucopyranoside (OG), and then treated with crystal violet. And Pool C was fixed with paraformaldehyde, permeabilized with the detergent, n-octyl-B-D-glucopyranoside (OG), and then treated with crystal violet. In the results published by Halden *et al.*, the third method performed on cell pool C was the most effective and it significantly decreased the background noise caused by the macrophage's natural autofluorescence. In the current studies the third method of fixing and permeabilizing the cells was slightly modified by substituting various concentrations of the quenching dye trypan blue for crystal violet. Unfortunately, with this method, the cells remained autofluorescent at all concentrations of trypan blue.

A second method tested was modeled closely after the trypan blue quenching method used by Moissman *et al.* Victoria L. Moissman and her colleagues successfully reduced the amount of autofluorescence in freshly isolated, antibody stained peripheral blood lymphocytes. In these studies, the cells were fixed and permeabilized with 1X Ortho Permeafix. The cells were then treated with various concentrations trypan blue (0.01  $\mu\text{g/ml}$ -2  $\mu\text{g/ml}$ ). Moissman and colleagues found that 2mg/ml of trypan blue significantly quenched the cellular autofluorescence without reducing the fluorescence intensity of the antibody-conjugated fluorescent probes. In the current studies, the protocol was slightly modified, as Cytoperm/Cytofix<sup>TM</sup> (Becton Dickinson) was used to fix and permeabilize the cells and higher

concentrations of trypan blue (ranging from 0.01 µg/ml-10 µg/ml) were tested. However, in these studies, the cells remained autofluorescent at all concentrations of trypan blue.

In our laboratory, the quenching of autofluorescence of the hybridoma cells, using models of both methods was unsuccessful. There were no significant differences between any amounts of trypan blue (including the absence of trypan blue) using either of the fixing and permeabilizing methods. Because the hybridoma cells were producing high amounts of autofluorescence coupled with the fact that the DX5 antibody is of low binding efficiency, we determined that antibody staining with FITC-conjugated DX5 antibody and subsequent flow cytometry analysis was not an efficient method for the detection of Natural Killer cell hybridomas.

In these studies, we were able to fuse freshly isolated lymphocytes with myeloma cells, but the identification of a pure NK hybridoma with the anti-DX5 antibody (biotin-conjugated rat anti-mouse Pan-NK cell monoclonal antibody) was not successful. In future studies, the problem of cellular autofluorescence in mixed lymphocyte cultures needs to be further investigated. Once this problem has been solved, other antibody staining protocols can then be employed in order to create a pure NK hybridoma clone pool.

Heberman R.B. *et al.* Natural Cytotoxic Reactivity of Mouse Lymphoid Cells Against Syngeneic and Alogeneic Tumors. I. *International Journal of Cancer*, 1975; 16(2): 216-229.

Karre, Klas. Express Yourself or Die: Peptides, MHC Molecules, and NK Cells. *Science*, 1995; 267: 978-979.

Kasai *et al.* A glycolipid on the Surface of Mouse Natural Killer Cells. *European Journal of Immunology*, 1980; 10:175-180.

Koo, G.C. and J. R. Peppard. Establishment of Monoclonal anti-NK-1.1 Antibody. *Hybridoma*, 1984; 3:301-303.

Kubota, Koichi *et al.* Generation of Novel Killer Hybridomas Derived from Proliferation –Supressed Somatic Cell Hybrids Between YACUT T Cell Lymphomas and Normal Lymphocytes Activated in Secondary Mixed Lymphocyte Cultures. *Journal of Immunological Methods*, 1996; 192: 137-147.

Kubota, Koichi, (1997) A Killer Cell Protective Antigen Expressed by MHC-Unrestricted Killer Hybridomas. *Cellular Immunology*, 1997; 181: 50-58.

Kuby, Janis. *Immunology*, 1997. Third Edition, W.H. Freeman and Company.

Lian R. H. *et al.* Recognition of Class I MHC by NK receptor Ly-49C: Identification of Critical Residues. *Journal of Immunology*, 1999; 162(12): 7271-6

- Mandy, Frances F. *et al.* Principles of Flow Cytometry. *Transfusion Science*, 1995; 16(4):303-314.
- Martin, Jose M. and E. Coen Buevery. Stability of Monoclonal Antibody Production in Cell Culture. *Flow Cytometry Applications in Cell Culture*, Marcel Dekker Inc., 1996.
- McKinney, Katherine L. and Georges Belfort. Use of flow Cytometry for Monitoring Antibody Productivity and Isolating High-Secreting Hybridoma Cells. *Flow Cytometry Applications in Cell Culture*, Marcel Dekker Inc., 1996.
- Mignari, M.C. *et al.* In Vitro Proliferation and Cloning of CD3-CD16+ Cells from Human Thymocyte Precursors. *Journal of Experimental Medicine*, 1991; 174(1): 21-26
- Moisman, Victoria L. *et al.* Reducing Cellular Autofluorescence in Flow Cytometry: An In Situ Method. *Cytometry*, 1997; 30:151-156.
- Moretta, A. *et al.* HLA-Specific and Non-HLA-Specific Human NK Receptors. *Current Topics in Microbiology and Immunology*, 1999; 244: 69-84.
- Moretta, L., Ciccone, E., Pende, D., Tripodi, G., Bottino, C., Moretta A., (1992) Human Natural Killer Cells: Clonally Distributed Specific Functions and Triggering Surface Molecules. *Laboratory Investigation*, 1992; 66 (2): 138-142.
- Morgan, S.J and D.C. Darling. *Animal Cell Culture: Introduction to Biotechniques*. First Edition, BIOS Scientific Publishers Limited.

- Nguyen Linh T. *et al.* Cross-Correlation for Flow Cytometric Histogram Background Subtractions. *Journal of Immunological Methods*, 2000; 238:151-160.
- Ortaldo J.R. *et al.* Ly-49 receptor Expression and Functional Analysis in Multiple Mouse Strains. *Journal of Leukocyte Biology*, 1999; 66(3):512-520
- Robertson, Michael J. and Jerome Ritz. Biology and Clinical Relevance of Human Natural Killer Cells. *Blood*, 1990; 76 (12): 2421-31.
- Sanchez, M.J. *et al.* Human Natural Killer Cell Committed Thymocytes and Their Relation to the T Cell Lineage. *Journal of Experimental Medicine*, 1993; 178(6):1857-66.
- Sivakumar, P.V. *et al.* Ontogeny and Differentiation of Murine Natural Killer Cells and Their Receptors. *Current Topics in Microbiology and Immunology*, 1998; 230: 161-190.
- Slifka, M.K. *et al.* NK Markers are Expressed on a High Percentage of Virus-Specific CD8+ and CD4+ T Cells. *Journal of Immunology*, 2000; 164(4): 2009-15.
- Suzuki, Yasumoto *et al.* Establishment of Hybridoma Cells wit Natural Killer (NK) Like Activity Against Syngenic Tumor Cells. *Microbiology and Immunology*, 1992; 36(4):401-406.
- Tuttles, Jill *et al.* Expression of Asialo-GM1 on a Subset of Adult Murine Thymocytes: Histological Localization and Demonstration that the Asialo-GM1-Positive subset contains both the Functionally Mature and the Proliferating Thymocyte Subpopulations. *The Journal of Immunology*, 1987; 138(2):364-372.

- Versteeg, R. NK Cells and T Cells: Mirror Images? *Immunology Today*, 1992; 13(7):244-7.
- Vicari, A. P. and A. Zlotnik. Mouse NK1.1+ T-cells: a New Family of T-cells. *Immunology Today*, 1996; 17: 71-76.
- Viksman, Michael Y. *et al.* Application of a Flow Cytometric Method Using Autofluorescence and a Tandem Fluorescent Dye to Analyze Human Alveolar Macrophage Surface Markers. *Journal of Immunological Methods*, 1994: 172:17-24.
- Wede, J. *et al.* Analysis of Single and Double Stained Alveolar Macrophages by Flow Cytometry. *Journal of Immunological Methods*, 1997; 207:115-123.
- Wigzell, H. and U. Ramstedt. Cellular Immunology; vol 2. *Handbook of Experimental Immunology*, 4<sup>th</sup> ed. Blackwell Scientific Publications, 1989.
- Wiltrout, Robert H. *et al.* Reactivity of Anti-Asialo GM1 Serum with Tumoricidal and Non-Tumoricidal Mouse Macrophages. *Journal of Leukocyte Biology*, 1985; 37:597-614.
- Yu, Y. Y. L. *et al.* Murine Natural Killer Cells and Marrow Graft Rejection. *Annual Review in Immunology*, 1992: 10:189-213.